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INFLUENCE OF LOCAL ABDOMINAL RADIATION ON RESPONSE OF RABBITS TO NEPHROTOXIC SERUM

R. B. ARHELGER, M.D.; P. N. SMITH, B.S.; B. L. WALKER, B.S.; W. M. FANT, M.D., AND J. G. BRUNSON, M.D.

From the Departments of Pathology and Radiology, University of Mississippi Medical Center, Jackson, Miss.

The administration of avian anti-rabbit kidney serum to rabbits results in proliferative glomerular lesions which resemble the morphologic features of human glomerulonephritis. Masugi [1] originally advanced the concept that these experimental lesions resulted from the reaction of the antiserum with antigenic sites present in the glomeruli. Kay [2] noted that this hypothesis did not explain the characteristic latent period between nephrotoxic serum injection and the development of the nephritis. He showed that exposure of the animals to total body radiation 3 days prior to the administration of nephrotoxic serum prevented the development of the glomerular lesions. On the basis of these studies, he suggested that the injected serum induced the formation of antibodies in the rabbit, and that these autologous antibodies then reacted with antigenic sites on the nephrotoxic serum antibodies which had localized in the glomerular capillaries. Lange, Wenk, Wachstein and Noble,[3] using the Coons fluorescent technique, demonstrated that there was an initial localization of the injected renal antiserum in glomerular capillaries and that this localized component later reacted with antibodies produced by the rabbit. These observations appeared to explain the latent period between serum administration and the appearance of nephritis.

Recently, Jarvis, Schrader and Brunson [4] described diffuse vascular fibrinoid lesions in rabbits given local abdominal radiation in conjunction with an intravenous injection of Gram-negative bacterial endotoxin, and suggested that local radiation might have systemic effects.

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In an attempt to obtain further information concerning the possible systemic effects of local abdominal radiation and to determine the pathogenesis of glomerular proliferative lesions based on antigen-antibody reaction, groups of rabbits were given nephrotoxic serum in conjunction with local abdominal radiation. This paper describes the morphologic alterations observed by light and electron microscopy in these animals.

Material and Methods

Nephrotoxic serum was produced in chickens by the repeated intraperitoneal injection of a rabbit kidney homogenate prepared according to the method described by Heymann and Lund.[5] Serums from several chickens were pooled, and the potency of the material was standardized. It was found that an intravenous injection of 3 ml. of the pooled serum produced diffuse glomerular proliferative lesions in the kidney examined 7 days after administration. Therefore, this dosage was used in all subsequent experiments.

A total of 128 hybrid albino rabbits of both sexes, each weighing approximately 1.5 kg., was used in the study. They were fed Purina rabbit pellets and had free access to water.

Animals given radiation were secured to an animal board with their abdomens facing a cobalt 60 source. The field of radiation covered both kidneys, the adrenal glands, and portions of the intestinal tract. Single depth doses of 570 r, 1,140 r, or 2,280 r were given in conjunction with a single intravenous injection of 3 ml. of nephrotoxic serum. The latter was given simultaneously with radiation or 18 hours prior to exposure. In other groups the serum was given 18 or 36 hours following radiation. Appropriate radiation and serum controls were used.

The rabbits were killed 7 days after the administration of nephrotoxic serum, and necropsy examinations were performed. Sections from the heart, lung, liver, spleen, kidney, adrenal gland, and gastrointestinal tract were fixed in 10 per cent neutral formalin. These were prepared in the usual manner for light microscopy and were stained with hematoxylin and eosin. Many additional sections of kidneys were stained by the periodic acid-Schiff (PAS) method. Sections from selected kidneys were cut into 1 mm. cubes, fixed in 1 per cent buffered osmium tetroxide for 2 hours, dehydrated in graded alcohols, embedded in a 9:1 mixture of butyl and methyl methacrylate, and sectioned with a glass knife on a Servall Porter-Blum microtome. Suitable thin sections were mounted on copper specimen grids and examined in an RCA EMU-3F electron microscope. Serial sections 1 to 2 μ thick were also cut from the methacrylate blocks and stained with Wright-Giemsa stain [6] or a modification of the PAS method [7] for light microscopy.

Results

The kidneys were the only organs which showed morphologic alterations.

Light Microscopy

As shown in Table I, 13 of the 16 control rabbits given serum alone showed renal glomerular alterations. These changes consisted of glomerular enlargement associated with extensive endothelial proliferation (Fig. 1). In many instances there appeared to be coalescence of capillary loops and lobulation of the glomeruli. When radiation was

given alone, no proliferative lesions were observed, although there was considerable nuclear pyknosis.

Proliferative changes were present also in 34 of 36 animals given serum 18 hours before, or simultaneously with, abdominal radiation. In addition, marked basement membrane thickening was observed in glomeruli from animals given simultaneous radiation and serum (Fig. 2).

When radiation preceded the administration of nephrotoxic serum, the results depended on the dosage of radiation and the interval between radiation and serum injection (Table II). Diffuse proliferative lesions similar to those of the serum control animals were present when 570 r was given 18 or 36 hours prior to serum, and extensive changes were observed also in the group exposed to 1,140 r 18 hours before serum injection. However, kidneys from rabbits given 1,140 r 36 hours prior to serum showed minimal or only focal areas of glomerular endothelial proliferation (Fig. 3). Similar results were obtained by giving 2,280 r 18 hours before serum.

When 2,280 r was given 36 hours prior to serum administration, only

TABLE I

RENAL LESIONS IN RABBITS RADIATED AFTER SERUM

Radiation dosage	Interval after serum	No. of animals	No. with glomerular lesions
570 r	Simultaneous	4	4
	18 hr.	8	7
1140 r	Simultaneous	4	4
	18 hr.	8	8
2280 r	Simultaneous	4	4
	18 hr.	8	7
Serum controls		16	13
Radiation controls		24	0

TABLE II

RENAL LESIONS IN ANIMALS RADIATED PRIOR TO SERUM

Radiation dosage	Interval before serum (hr.)	No. of animals	No. with glomerular lesions
570 r	18	8	8
	36	4	4
1140 r	18	8	7
	36	4	3 (Minimal to variable)
2280 r	18	8	7
	36	20	1

one of 20 rabbits developed minimal glomerular proliferative lesions. The remaining 19 showed no evidence of glomerular hypercellularity or basement membrane thickening (Fig. 4).

Electron Microscopy

The most prominent feature observed in glomeruli from rabbits given nephrotoxic serum alone was proliferation of capillary endothelial cells. The endothelial cytoplasm appeared swollen, and frequently the increased size and number of endothelial cells resulted in complete obliteration of capillary lumens (Fig. 5). The epithelial cells were somewhat swollen also, and their foot processes often coalesced to form a continuous layer of cytoplasm extending along the outer surface of the basement membrane (Fig. 6). The capillary basement membranes were of normal thickness in all of the sections examined.

Similar endothelial and epithelial alterations were observed in the groups given serum prior to all dosages of radiation, in those given 570 r 18 or 36 hours before serum, in glomeruli from animals given 1,140 r 18 hours prior to serum, and when radiation and serum were given simultaneously.

In the groups given simultaneous radiation and serum, the apparent basement membrane thickening observed by light microscopy was seen to be the result of deposition of a dense, finely granular substance between the basement membrane and the endothelium (Fig. 7). This substance was occasionally seen also within epithelial cytoplasm. Light microscopic examination of adjacent sections cut at 1 to 2 μ and stained by a modification of the PAS method, as well as sections from paraffin embedded tissue, showed this material to exhibit the morphologic and tinctorial characteristics of fibrinoid.

Electron microscopy of glomeruli from rabbits given 1,140 r 36 hours before or 2,280 r 18 hours before serum substantiated the light microscopic observations. Although endothelial proliferation was present, this alteration was much less extensive than in control animals. No glomerular changes were present in sections from rabbits exposed to 2,280 r 36 hours prior to nephrotoxic serum administration (Fig. 8).

Discussion

The results indicate that local abdominal radiation in a dosage of 2,280 r given 36 hours prior to the injection of a nephrotoxic serum prevented the development of glomerular endothelial proliferation in a high percentage of rabbits. When the same dose was given 18 hours before serum, or when 1,140 r was given 36 hours before serum, the lesions were present, but were less extensive than in the glomeruli from serum

control animals. However, the development of the lesions was not inhibited by exposure to 570 r or 1,140 r 18 hours prior to nephrotoxic serum administration, and extensive lesions were present also when serum injection preceded radiation.

It is interesting to note that glomerular capillaries from animals given simultaneous radiation and serum showed the subendothelial deposition of fibrinoid substance, similar to that previously described in glomeruli in rats given nephrotoxic serum in conjunction with Gram-negative endotoxin.[8] In those studies it was shown that a cold heparin-precipitable fraction (presumably altered fibrinogen), which has been demonstrated in the plasma of normal rats,[9] was not present following injection of nephrotoxic serum alone.

Fibrinoid deposition has been described also by Jarvis and coworkers[4] in glomeruli from rabbits given local abdominal radiation in conjunction with endotoxin. The development of the lesions was shown to correlate with a decrease in plasma fibrinogen[10] in a manner similar to that seen during the development of the generalized Shwartzman reaction.[11]

In 1954, Kleinerman[12] showed that nephrotoxic serum nephritis in rabbits could be inhibited by heparin administration. He suggested that heparin prevented the final antigen-antibody reaction by destruction or alteration of complement. These observations indicate that a study of fibrinogen levels and the effects of heparin administration in rabbits given simultaneous radiation and nephrotoxic serum might provide further information regarding the pathogenesis of renal vascular fibrinoid lesions.

The mechanism by which local abdominal radiation prevents the development of a glomerular lesion based on an antigen-antibody reaction is not as yet clear. It is possible that local radiation exerts a systemic effect which inhibits the development of antibodies by the rabbit. Another possibility is that local radiation prevents the reaction between injected renal antiserum which has localized in the glomeruli and antibodies developed by the rabbit, possibly by alteration of complement.

Preliminary studies utilizing the fluorescent antigen-antibody technique have demonstrated localization of the antiserum in rabbit glomeruli 24 hours after injection. However, when rabbits were exposed to 2,280 r 36 hours prior to serum administration, there was no demonstrable localization of antiserum. Therefore, it appears more probable that radiation inhibits the localization of antiserum in the glomerular capillaries, thereby preventing a subsequent antigen-antibody combination in the glomeruli between the bound antiserum and the autologous antibodies.

Summary

Rabbits were given local abdominal radiation and nephrotoxic serum simultaneously or separately with varied intervals between radiation and serum injection. Control animals were given radiation or serum alone. Rabbits given nephrotoxic serum alone developed extensive glomerular endothelial proliferation. Glomeruli from animals given 570 r or 1,140 r 18 hours before serum showed similar changes, and extensive lesions were present also when serum injection preceded radiation. In addition to endothelial proliferation, fibrinoid deposition between basement membranes and endothelium and within epithelial cytoplasm was observed in sections from animals given simultaneous radiation and serum.

When 1,140 r was given 36 hours prior to serum injection, or when 2,280 r was given 18 hours before serum, glomerular proliferative lesions were observed but were much less extensive than in control animals given nephrotoxic serum alone. Only one of 20 rabbits exposed to 2,280 r 36 hours prior to serum administration developed minimal proliferative changes. The remaining 19 showed normal glomerular structure with no evidence of endothelial proliferation, foot process smudging, or fibrinoid deposition.

The results suggest that local abdominal radiation acts to prevent the development of nephrotoxic serum nephritis in rabbits by inhibiting localization of antiserum in the glomerular capillaries, thereby preventing a later reaction between the bound antiserum and autologous antibodies developed by the rabbit.

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We gratefully acknowledge the technical assistance of Mrs. Shirley Long.

[*Illustrations follow*]

Legends for Figures

All sections were taken 7 days after injection of nephrotoxic serum.

Key:

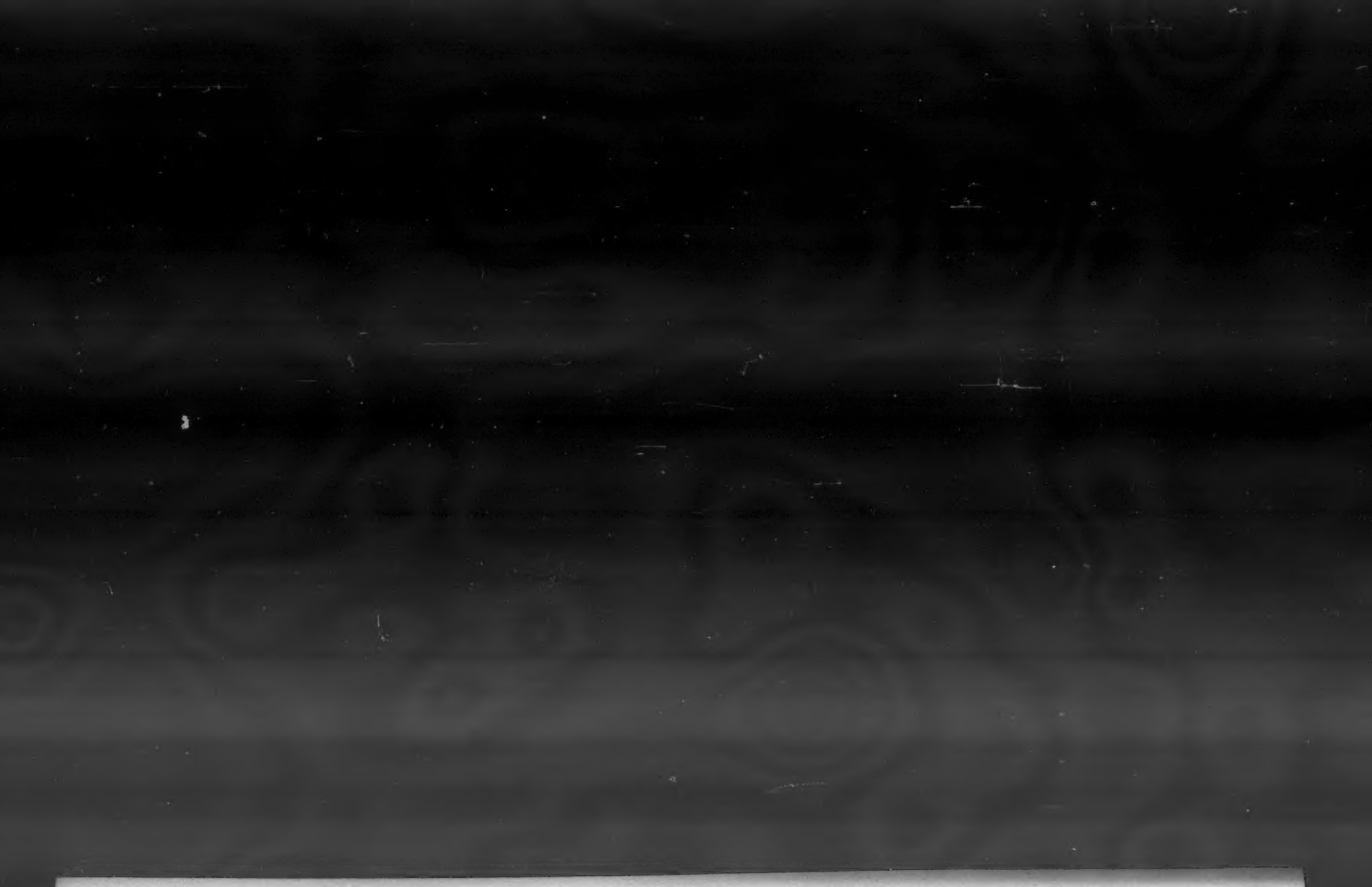
End = Endothelium
Ep = Epithelium
BM = Basement membrane
F = Coalesced epithelial foot processes
CL = Capillary lumen
fib = Fibrinoid
RBC = Red blood cell
WBC = Leukocyte

Fig. 1. Enlarged, lobulated glomerulus showing marked endothelial proliferation. Rabbit given 3 ml. of nephrotoxic serum alone. Hematoxylin and eosin stain. × 480.

Fig. 2. Simultaneous radiation (1,140 r) and serum. Methacrylate-embedded (2 μ) serial section from glomerulus shown in Figure 6. Note thickened PAS-positive capillary membranes and a moderate degree of endothelial proliferation. Modified PAS stain. × 960.

Fig. 3. Glomerulus from a rabbit given 1,140 r 36 hours prior to serum. Note open capillaries which contain red cells and show minimal endothelial proliferation. Hematoxylin and eosin stain. × 600.

Fig. 4. Glomerulus from a rabbit given 2,280 r 36 hours before serum. Note normal size of the glomerulus, open capillary loops, and the lack of proliferative changes. Hematoxylin and eosin stain. × 640.



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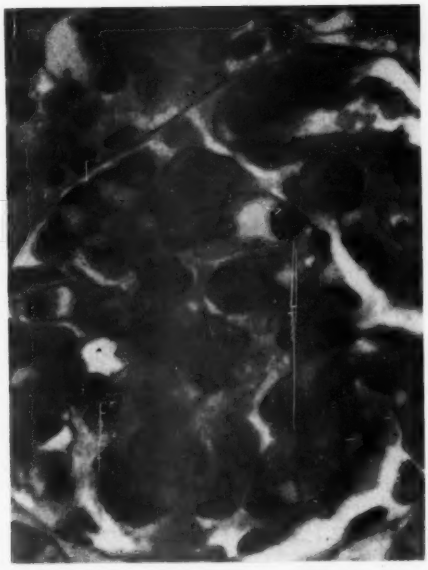

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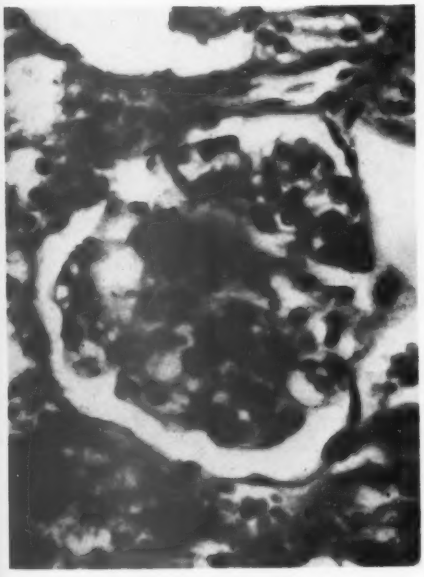

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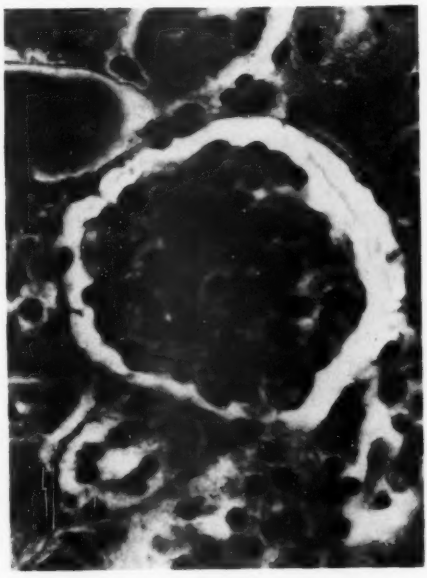

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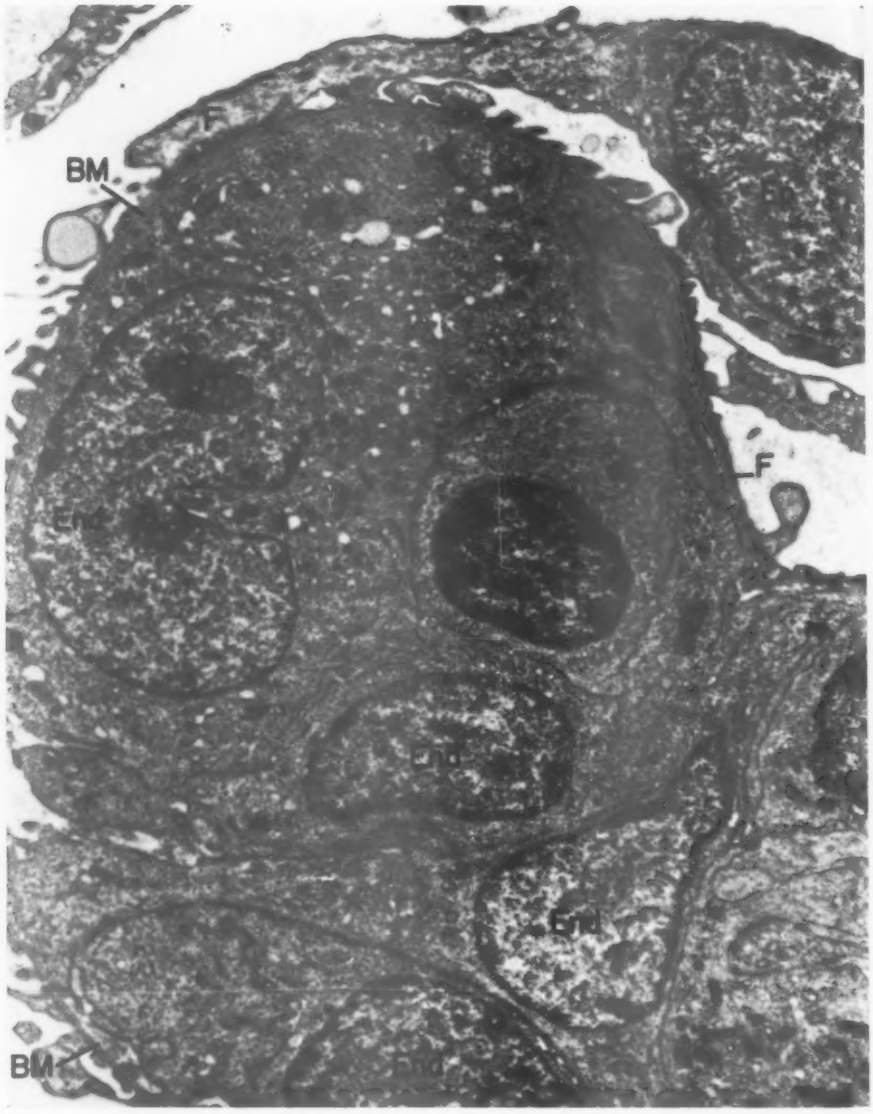
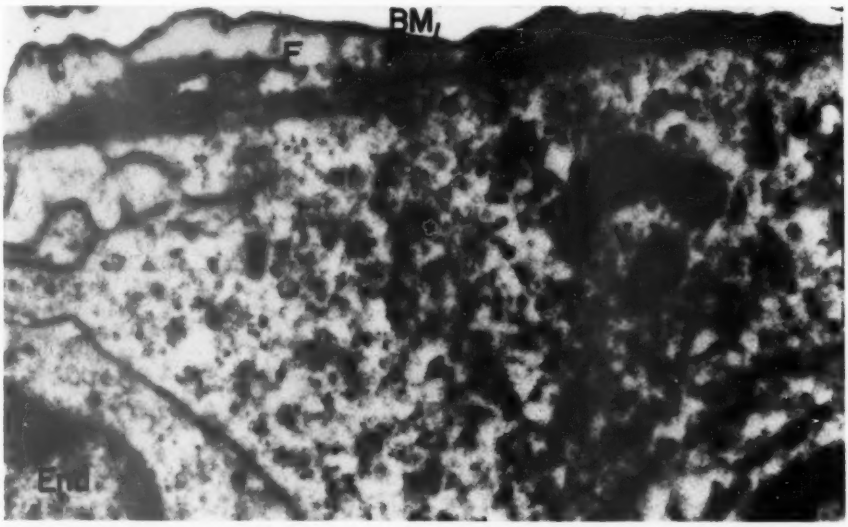


FIG. 5. A glomerular capillary loop from a rabbit given 3 ml. of nephrotoxic serum alone. Note complete obliteration of the lumen by endothelial swelling and proliferation. The basement membrane is of normal thickness, but in two areas the epithelial foot processes are broadened and have lost their normal discrete structure. × 9,900.



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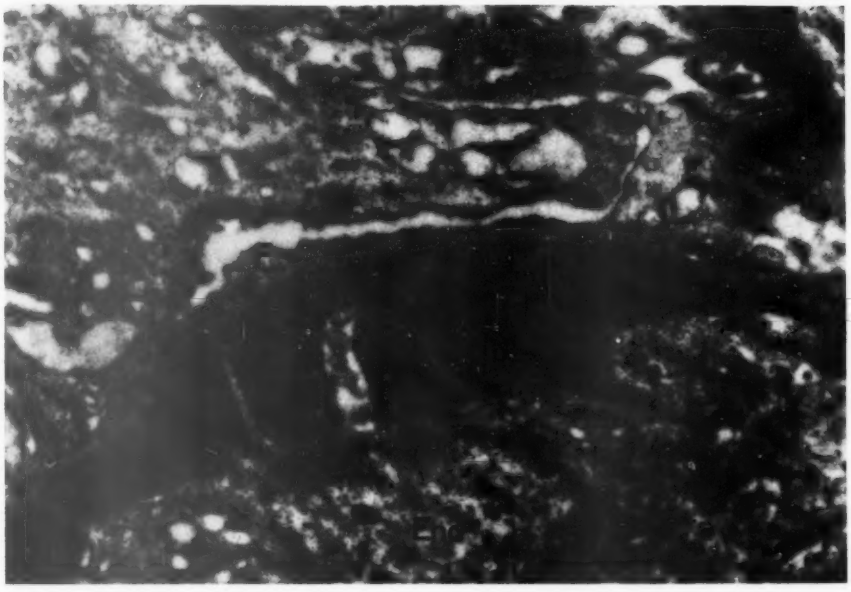

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FIG. 6. A glomerular capillary wall from the same animal, showing epithelial foot process smudging. Again, note normal thickness of the basement membrane. Portions of 3 endothelial cells are illustrated. × 23,000.

FIG. 7. A glomerular capillary wall from a rabbit given simultaneous radiation (1,140 r) and nephrotoxic serum. Dense, finely granular fibrinoid substance is seen between the basement membrane and the endothelium, and also within the epithelial cytoplasm. The epithelial foot processes have lost their normal structure and form a continuous layer of cytoplasm along the outer aspect of the basement membrane. × 18,200.

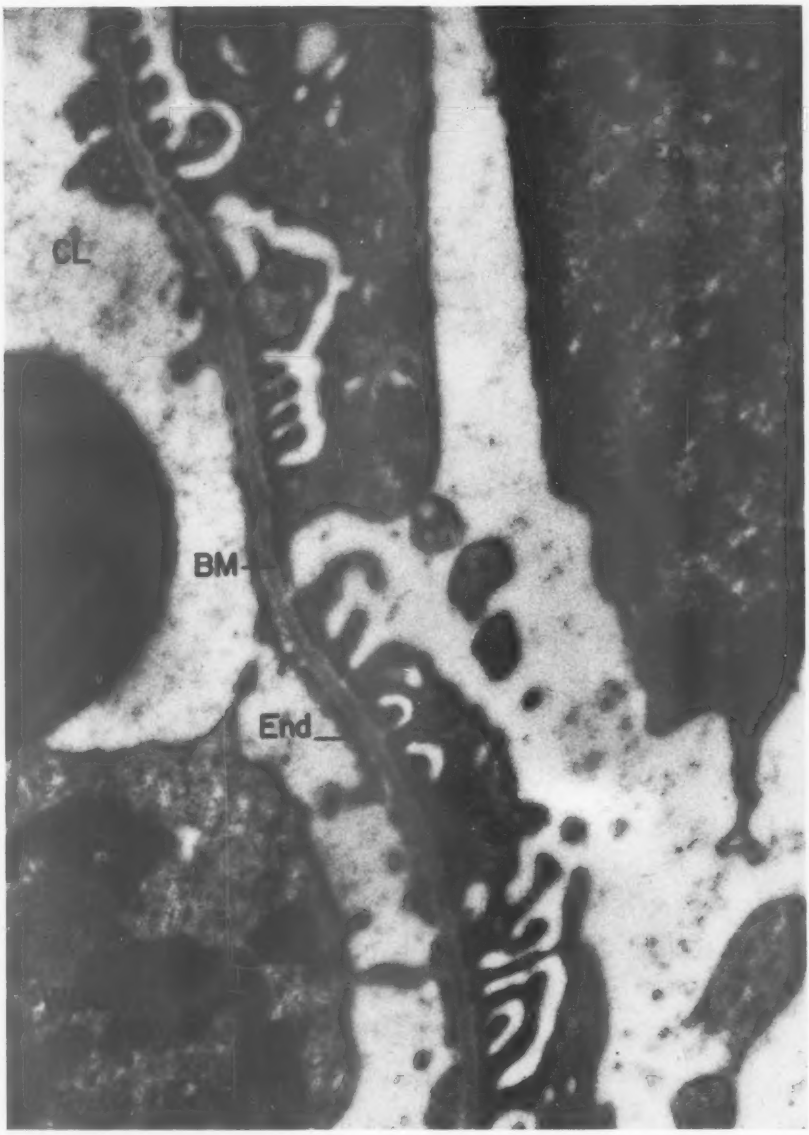
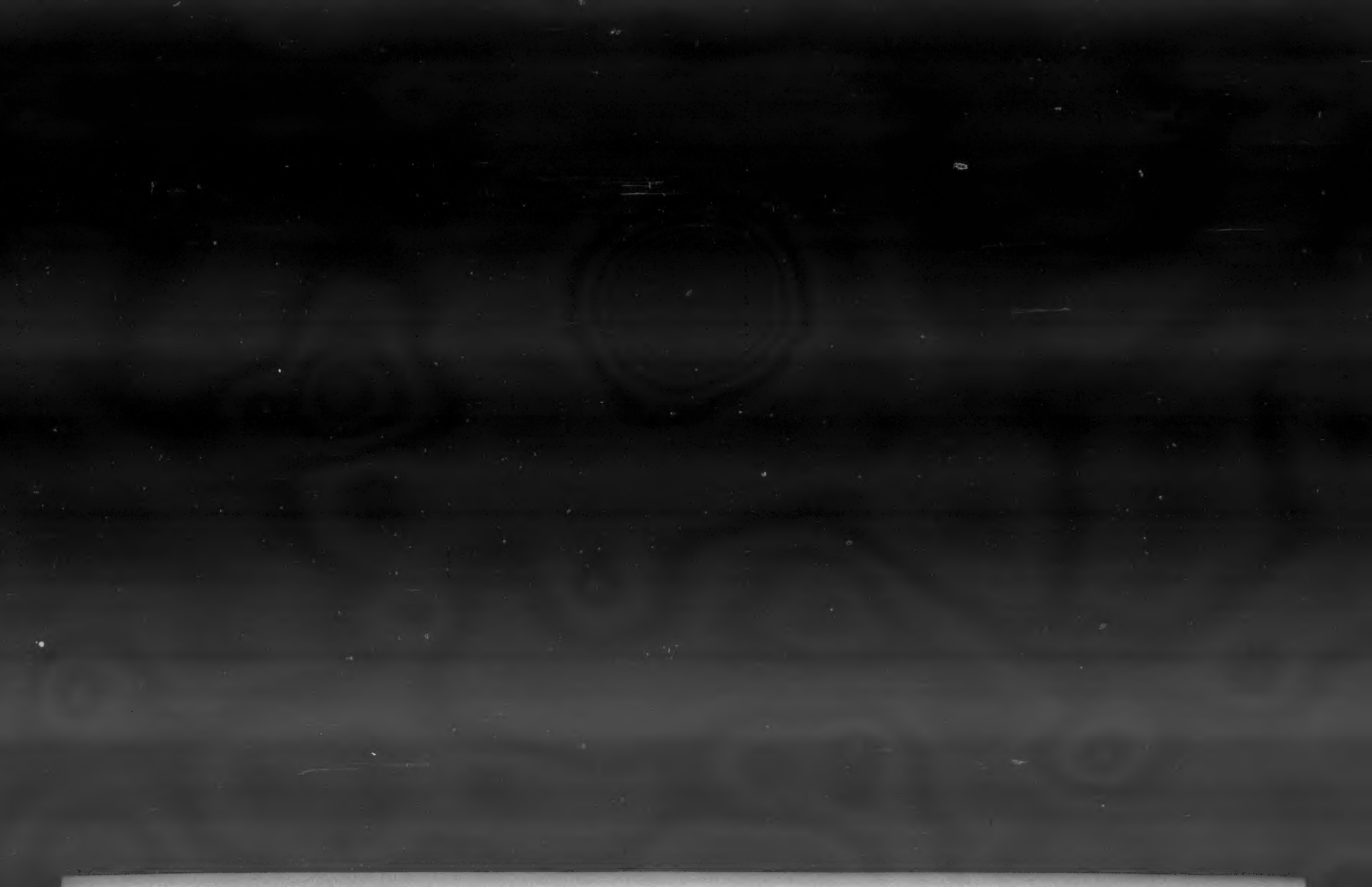


FIG. 8. A glomerular capillary wall from an animal given 2,280 r 36 hours before serum, showing normal glomerular structure. Note the open capillary lumen containing a red cell and a leukocyte, the thin endothelial lining with its fenestrations, the normal thickness of the basement membrane, and the epithelium with discrete foot processes. × 27,000.



ELECTRON MICROSCOPIC STUDIES ON THE DEVELOPMENT OF THE GLOMERULAR LESIONS IN AMINONUCLEOSIDE NEPHROSIS

JAN L. E. ERICSSON, M.D., AND GIUSEPPE A. ANDRES, M.D.*

From the Department of Pathology II, Karolinska Institutet, Sabbatsberg's Hospital, Stockholm, Sweden

The etiology and pathogenesis of the nephrotic syndrome in man have long been debated. Despite great progress in experimental and clinical research in recent years, however, there remain many unsolved problems. It is now well known that this syndrome may occur during the course of a variety of diseases with different etiology; i.e., diabetes mellitus, amyloidosis, lupus erythematosus. On the other hand, in many instances the nephrotic syndrome develops as an isolated condition with unknown cause. This latter group has attracted much attention from both the clinical and morphologic points of view. Many recent reports of renal biopsy studies in such cases have been concerned with the value of electron microscopy and thin sections for light microscopy. These have shown that the earliest lesions were localized in glomeruli [1–5]; the varied pattern of the glomerular alterations (proliferative and membranous glomerulitis and mixed forms) even in early stages, however, has made it difficult to correlate the morphologic features with available clinical data. There appears to be no pathognomonic lesion in the glomerulus. Moreover, it has been even more difficult to investigate sequential glomerular changes in cases with long drawn-out courses ending in renal failure.

For the purpose of procuring more detailed knowledge of the early lesions and their progression into the chronic phase with renal failure, many attempts have been made to produce the nephrotic syndrome in the experimental animal. It was not until 1955, however, that Frenk, Antonowicz, Craig and Metcoff [6] published the method of producing acute nephrosis in the rat through repeated injections of aminonucleoside, thus making it possible to induce the disorder experimentally. Since this report there have been many publications concerning the clinical, patho-anatomic and biochemical changes in aminonucleoside nephrosis.[7–19] Most of these have given descriptions of the acute lesions occurring during the first 2 to 4 weeks only. Electron microscopic studies of the kidneys have shown early alterations in the glomerular epithelium. Farquhar and Palade [15] presented direct evidence that the epithelial damage was due to a primary defect in the basement membrane.

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* Fellow, Svenska Institutet and Ministero Italiano Esteri, 1959.

Attempts to induce a long drawn-out course similar to that seen in man have often been unsuccessful. It has been difficult to keep the animals alive for any longer time period without a diminution in the manifestations of nephrosis. Some authors [8-10,17,18] have reported the appearance of chronic renal disease with renal failure several months after the inception of aminonucleoside injections. However, to our knowledge, only the article by Feldman and Fisher,[20] which appeared when this manuscript was completed, has been concerned with the progression of acute aminonucleoside nephrosis into a chronic phase. In a detailed ultrastructural analysis of the sequence of pathologic changes, Feldman and Fisher described progressive glomerular lesions characterized by thickened glomerular basement membranes, adherence between capillary and Bowman's capsule, deposits of basement membrane-like material in and around the capillaries, and thickening and splitting of Bowman's capsule.

In our experiments a nephrotic syndrome was produced in the rat by subcutaneous injections of aminonucleoside for 6 to 15 days. The nephrosis was then maintained by intravenous injections, and it was possible to study the evolution of the disease for a period up to one and a half years.

The aim of this study has been to give a description of the clinical data and the morphologic renal changes during various stages of the nephrosis.

Material and Methods

Twenty rats of the Sprague-Dawley strain, weighing 70 to 90 gm., were used. They were maintained on a regular laboratory rat diet (Rockland Farms, New York City) and water *ad libitum*.

All animals received a daily subcutaneous injection of a 0.5 per cent aqueous solution of aminonucleoside (6-dimethylamino-purine-3-amino-d-ribose)* in a dose of 0.003 ml. per gm. of body weight. After 14 to 16 injections a complete nephrotic syndrome developed.

Eleven animals were killed during the acute stage of the disease, 6 to 26 days after the beginning of the experiment. In 9 rats a chronic disease was produced by giving intravenous injections of 0.5 per cent aminonucleoside solution (1 to 1.5 ml.) as soon as proteinuria had subsided (generally 1 injection every second month). These animals were killed at various intervals after 22 to 78 weeks by heart bleeding performed under general ether anesthesia. Controls were killed simultaneously.

The kidneys were removed immediately and fixed in Bouin's solution for light microscopy and in osmium tetroxide (Palade[21]) for electron microscopy.

Quantitative urinary proteins were determined daily in the acute cases and weekly in subchronic or chronic cases.[22] Nonprotein nitrogen (NPN) was determined by the van Slyke micro-Kjeldahl method. The systolic blood pressure was recorded, utilizing a photoelectric tensometer.† In our control rats the average systolic blood pressure ranged from 100 to 120 mm. of Hg (average value of 6 to 10 controls). The

* Kindly supplied by Lederle Laboratories Division, Pearl River, N.Y.

† Metro Industries, Long Island City, N.Y.

presence and extent of edema and ascites were also recorded. Animals were weighed daily and those receiving aminonucleoside revealed a gain in body weight similar to that in untreated controls despite the presence of edema and ascites.

Renal tissue prepared for light microscopy was stained by the hematoxylin and eosin, van Gieson, Mallory, and periodic-acid-Schiff (PAS) methods. The tissue fixed in 1 per cent buffered osmium tetroxide for 1½ to 2 hours in an ice bath was rinsed in buffer, dehydrated in serially increasing concentrations of alcohol and embedded in a 4:1 mixture of butyl and methyl methacrylates. Sections were cut with a Leitz ultra microtome and examined with RCA EMU 2d or Siemens Elmiskop I electron microscopes. For orientation, 0.5 to 1 μ sections were examined by phase contrast microscopy after the methacrylate had been dissolved with amyl acetate. The latter sections were procured immediately after those for electron microscopy. In this way it was possible to compare the electron micrographs and conventional microscopy in the same locality. Some of the sections taken for phase microscopy were also stained with crystal violet and basic fuchsin.[22]

Experimental Results

In rats with the acute disorder the clinical results were essentially similar to those reported by others.[13,16] There developed a nephrotic syndrome with edema and heavy proteinuria. In none of the animals were there any signs of renal failure with nitrogen retention.

The bi-monthly intravenous injections of aminonucleoside produced a chronic disease continuing as long as 1½ years (Table I). In this way in most of the animals, proteinuria was maintained at a high level, and there was also edema. However, after 5 to 10 months there was a change in the clinical pattern. Edema diminished or vanished, and in some instances the blood pressure rose and evidence of increasing renal failure with elevated NPN values appeared. In the two animals surviving for 1½ years it was possible to study the full development of the chronic disorder, ending with uremia and hypertension.

Macroscopically the kidneys in animals with acute disease were large, pale and swollen. In the two chronic cases, on the other hand, the kidneys were small and contracted, with granulated surfaces.

Light Microscopy

In most of the animals with acute disease (from the sixth injection up to about 1 month) the alterations were very slight. Glomeruli appeared larger than normal, often entirely filling the capsular space. Many capillary loops were obliterated by endothelial swelling, and very few red cells were found in the capillary lumens (Fig. 1). It was often possible to detect groups of intra-epithelial PAS-positive granules. Occasionally, also, there was a slight widening of the PAS-positive zone corresponding to the basement membrane and adjacent portions of epithelial and endothelial cytoplasm. About 2 weeks after the first injection, most of the glomeruli were hypercellular. The tubules appeared normal or showed slight to moderate widening, with hyaline casts.

TABLE I

CHRONIC AMINONUCLEOSIDE NEPHROSIS

Rat no.	Time after first injection (wk.)	No. of injections S.C.*	No. of injections I.V.*	Time of sacrifice (wk.)	Urinary protein (gm. %)	NPN (mg. %)	Blood pressure	Edema
12	4	15	2	25	2.8	26	110	++
	8				1.4			+
	12				0.3		122	++
	16				2.0	34		+
	20				3.0		144	+
	24				4.2	42	156	0
13	2	15	3	27	3.2	22	102	+
	4				1.8			
	8				2.4	32	102	++
	12				0.6			
	16				2.1			
	20				2.4	40	115	0
	24				1.2			
	27				0.4	30	119	0
14	2	15	3	22	3.2			
	4				1.8		115	++
	8				2.6	26		
	12				3.2		122	0
	16				1.6	24	142	0
	18				2.3	38		0
	20				1.0			
	22				1.0	30	129	0
15	4	15	3	28	6.8	24	98	++
	8				3.2	30	108	++
	10				6.0			
	12				3.8	26	119	+++
	20				4.4	42	140	+
	24				2.2	56		++
	28				3.6	58	149	0
16	4	15	3	30	1.5	22	92	+
	8				2.2			++
	12				3.8	22	100	
	16				1.7		100	+
	20				2.0	36	115	+
	24				3.2		129	0
	28				2.4	44	118	0
17	4	15	3	40	3.0	24	110	++
	12				5.0			
	16				4.0		122	+
	20				0.8	42		
	26				1.8		115	0
	32				2.0	44		0
	40				0.7	58	115	0
18	4	15	3	48	2.2	22	125	++
	12				3.0			
	17				1.7		105	+
	26				1.3	34		
	38				2.2	50	115	0
	48				1.7	42	125	0

TABLE I (*Continued*)

CHRONIC AMINONUCLEOSIDE NEPHROSIS

Rat no.	Time after first injection (wk.)	No. of injections S.C.*	No. of injections I.V.*	Time of sacrifice (wk.)	Urinary protein (gm. %)	NPN (mg. %)	Blood pressure	Edema
19	4	15	6	74	5.0	24	119	++
	8				3.2		127	+
	12				10.4			
	22				5.8	42	105	++
	26				6.2			
	32				2.0	38	125	+
	36				1.7			
	56				2.2	56	166	o
	65				1.0		210	o
	74				2.1	122	234	o
20	4	15	7	78	3.2	22	105	++
	12				2.4		115	+
	22				1.6		125	+
	32				0.8			
	36				2.4	40		+
	52				3.2	36		
	56				3.8			o
	61				6.4	88	163	
	70				5.2			o
	74				2.0	92		o
	78				1.7	89	190	o

* S.C. = subcutaneous injection; I.V. = intravenous injection.

During the development of the chronic phase, the glomerular lesions in animals killed after 5 to 10 months were entirely different from those observed during the acute stage. These consisted of an irregular thickening of the capillary and capsular basement membranes, most apparent in the loops adherent to the capsule. Here there seemed to be a fusion between the capillary wall and the capsule, with the formation of a continuous hyaline mass. In the most severely damaged glomeruli there was a nearly complete destruction of the capillaries, with no patent loops and large broad bands of hyaline, PAS-positive material. The lesions were unevenly distributed, and in the same kidney some glomeruli showed very severe damage while others were unaffected. Crescent formation with focal proliferation of the capsular epithelium was rarely encountered.

In the latest stages (more than 10 months' duration) hyalinization of the glomeruli was widespread and more evenly distributed, affecting the majority of the glomeruli (Fig. 2). In all cases attaining the chronic phase, tubules in both cortex and medulla showed flattened epithelial dilatation and contained hyaline casts or cell detritus. The interstitial

tissue exhibited focal inflammation, with infiltration by plasma cells and lymphocytes and increase of interstitial connective tissue.

Phase contrast microscopy showed swelling of tubular epithelial cytoplasm during the acute and chronic phases (Fig. 1). In the proximal convoluted tubules the swelling was usually focal and occasionally the cytoplasm appeared empty or contained only a few scattered mitochondria and protein absorption droplets. The focal cytoplasmic swelling caused a bulging of the cell content into the tubule lumen which occasionally was filled with such material.

Electron Microscopy

Acute Phase. The glomerular changes were essentially as described by other authors. There was distortion of foot processes along the basement membrane (Figs. 3 and 5). The epithelium appeared swollen and contained varying numbers of vacuoles, either empty or filled with granular or hyaline substance with varying electron density. These features were encountered even in a rat with very slight proteinuria after only 6 injections of aminonucleoside.[13] After 2 weeks, epithelial alterations were pronounced, and by this time there was also endothelial damage with cytoplasmic swelling causing focal obliteration of the capillary lumen (Fig. 5). Some glomeruli were hypercellular by reason of endothelial proliferation. There was no or only very slight focal swelling and increased thickness of the basement membrane. It was not possible to find deposition of a "foreign" substance in the endothelial cytoplasm or on the endothelial side of the basement membrane. However, in the fused and flattened epithelial cell foot processes outside the basement membrane a dark granular zone was often noted (Figs. 3 and 5).

Chronic Phase. In cases with persisting proteinuria after 5 to 10 months, proliferation, enlargement and swelling of the endothelium, and droplet formation, foot process alterations and vacuolation of the epithelium were as severe as in the acute stage (Figs. 6 and 7). In cases with diminishing proteinuria these changes regressed in varying degrees.

In all rats with the chronic lesion (except rats 13 and 14, in which it was not possible to produce a proper nephrotic syndrome despite intravenous injections of aminonucleoside) a special type of lesion was observed. This consisted of a diffuse thickening, with wrinkling and irregular outpouchings of the basement membrane ("spurs"), mainly on the endothelial side and lifting up the endothelial cytoplasm (Figs. 7 and 8). The content of the "spurs" usually had the same density and appearance as the basement membrane proper but occasionally appeared somewhat lighter or granular. Further development consisted of elongation and widening of the basement membrane outpouching with the forma-

tion of interdigitating broad trabeculae in contact with the membrane and small surrounding islands of endothelial cytoplasm (Fig. 10). In late stages endothelial cells were completely replaced by this substance which also brought about an obliteration of the capillary lumen. Epithelial cells were ordinarily unaffected by this basement membrane thickening until very late in the disease. The hyaline substance in the thickened membrane and its outpouchings was clearly separated from both endothelial and epithelial cytoplasm during the development of glomerular hyalinization. The substance invariably had a structureless, "hyaline," or rarely granular appearance; no signs of collagen formation were ever found.

In Bowman's capsule there were focal or diffuse thickenings of the basement membrane and swelling, enlargement and occasionally proliferation of the capsular epithelium (Fig. 9). In addition, in instances where crescent formation was observed, there was also splitting of the basement membrane (Fig. 10) and a marked proliferation of the capsular epithelium. Between these proliferating cells, hyaline basement membrane-like strands were deposited.

The two rats (Table I, rats 13 and 14) that developed the acute nephrotic syndrome but failed to show progression to the chronic phase despite intravenous injections of aminonucleoside showed no lesions in the glomeruli after 22 and 27 weeks respectively.

Discussion

Many of the investigations concerned with the induction of the acute nephrotic syndome in the rat by aminonucleoside have provided descriptions of the electron microscopic features in renal lesions, especially the glomeruli. In general there has been agreement among the various authors concerning the ultrastructural glomerular features; differences seem to be due mainly to variations in the amount of aminonucleoside given. Thus, those who had given daily subcutaneous doses larger than 0.5 mg. per 100 gm. of body weight reported that most of the animals failed to survive more than 12 to 14 injections.[11,14] Animals receiving such high doses died in uremia and had more pronounced glomerular lesions than those given smaller doses. With the dose used by us (0.3 mg. per 100 gm. of body weight of a 0.5 per cent aqueous solution), it was possible to induce the nephrotic syndrome with a low mortality among the animals and without the development of uremia in the acute stage. The earliest detectable glomerular lesions in our investigation appeared on the sixth day.

Similar features have been described by other authors in patients and experimental animals with proteinuria and are considered sequelae to the leakage of protein through the glomerular capillary wall. In our

experiments the lesions appeared after 6 days and before the onset of heavy proteinuria. The probable explanation is that increased glomerular filtration of protein is not immediately detectable in the urine because the tubules have the ability at the outset to reabsorb all the protein. Later the tubules become saturated or damaged and can no longer reabsorb the surplus protein; proteinuria results. This hypothesis is further supported by the existence of so-called protein-absorption droplets in the tubules simultaneously with the earliest glomerular changes. Our observations are in agreement with those of Harkin and Recant[16] and Feldman and Fisher[13] who also found glomerular alterations prior to proteinuria.

Aside from foot process alteration, a prominent early feature of the glomerular epithelial cytoplasm was the appearance of vacuoles, empty or filled with a substance of varying electron density (Figs. 3 and 4). This was usually very dark, homogeneous, and surrounded by a more or less prominent monolayered membrane. However, transitional forms between "dense bodies" and empty vacuoles were characterized by vacuoles containing finely granular material. Our investigation supports the view put forward by Farquhar and Palade[15,24,25] that these bodies and vacuoles are the morphologic reflection of an increased passage of proteins through the glomerulus; they paralleled the amount of proteinuria and disappeared when this was terminated. Moreover, Post[26] recently observed similar changes in glomerular epithelium after albumin loading, thereby affording additional support to this hypothesis.

The increased glomerular cellularity apparent after two weeks (Fig. 5) was attributable in the main to endothelial proliferation; endothelial cells were often found inside the capillary loops surrounded by basement membrane and epithelium. The existence of intercapillary or mesangial cells in glomeruli has been much debated, and for the moment the question of their existence must remain unanswered.

In the acute stage no obvious basement membrane thickening was observed, nor were basement membrane lesions similar to those described by Spiro, Caulfield and Craig[19] encountered. The basement membrane thickening observed by light microscopy at this stage is probably caused by fusion and swelling of epithelial foot processes and the accumulation of dark granular material in the cytoplasm of these cells.

Our investigations have shown that the changes in the glomerular epithelium and endothelium were potentially reversible since the glomeruli appeared entirely normal in the two rats in which manifestations of the acute nephrotic syndrome subsided despite the continued intravenous administration of aminonucleoside for 27 weeks.

Glomerular basement membrane lesions during the subacute stage of

aminonucleoside nephrosis (i.e., focal thickening and tortuosity) have been described by several authors.[16,19,20,27] We consider this thickening to be an essential feature in the development of the chronic disease. The formation of "spurs" in the early stages paralleled the diffuse thickening. These could be seen in both the axial and the peripheral portions of the loops, indicating their pathologic significance. The thickening was not the result of an extramembranous apposition of a foreign substance so far as could be judged by electron microscopy. The substance in both the thickened basement membrane proper and the hyaline strands replacing the endothelial cells usually had the same density and lack of structure as the normal membrane. Thus there is no clue as to the exact nature of the hyaline substance; it may be supposed that it is chemically closely related to the content of the basement membrane itself.

In discussing the causes of the glomerular lesions in the chronic stages, it is necessary to take into consideration the effect of hypertension. However, relatively pronounced glomerular basement membrane thickening and even partially hyalinized glomeruli were observed in rats with no hypertension. It is therefore probable that the hypertension was caused by the glomerular changes and not the reverse.

Ashworth, Erdmann and Arnold [28] have described age changes in the glomerular basement membrane of rats. They concluded that thickening of the dense, PAS-positive central zone of the membrane occurred with increasing age. In our experiments, young rats were used and the membrane thickening began as early as 5 to 6 months. Moreover, by that time, the thickening was far more pronounced in our animals than has been described in normal two-year-old rats. Thus, the effect of aging on the thickness of the basement membrane apparently had no significance in our experiments.

The sequence of glomerular changes in the progression of the renal disorder caused by aminonucleoside appears to be as follows: (1) During the early phase of the nephrotic syndrome (2 to 4 weeks), reversible epithelial and endothelial damage occurs, and there is proliferation, mainly of endothelium. (2) After 6 to 8 months there is irregular basement membrane thickening and partial hyalinization of some glomeruli. Clinically, a more or less severe nephrotic syndrome continues at this time, but there is no hypertension and normal or slightly elevated NPN values are observed. (3) The end stage supervenes after more than 10 months and is characterized by hypertension and uremia. The morphologic pattern is now that of widespread and pronounced renal damage, with hyalinization of most of the glomeruli and dilatation and atrophy of the tubules.

It is interesting to compare the experimental disease in the rat with

lipoid nephrosis in children and so-called membranous glomerulonephritis usually occurring in the adult. The clinical similarity is especially striking in membranous glomerulonephritis; this and the experimental disease may terminate in renal failure, with uremia and hypertension. However, the pathogenesis of the basement membrane thickening in the two conditions seems to differ. In membranous glomerulonephritis Farquhar [4] and Fiaschi, Andres, Giacomelli and Naccarato [5] have shown that the basement membrane thickening appears to reflect the apposition of a foreign substance on the epithelial side of the membrane. In contradistinction, the experimental disease is characterized by thickening on the endothelial side of the membrane. In this respect there is similarity between the chronic lesions in aminonucleoside nephrosis and those in children with progressive glomerular damage during so-called pure lipoid nephrosis. In the latter, Farquhar noted "an irregular nodular thickening of the homogeneous basement membrane . . . Along with this process there was a slight increase in the number of endothelial cells present, and finally the deposition between endothelial cells of material resembling basement membrane. With extension of these processes, a glomerulus became entirely obliterated by a combination of basement membrane thickening, mild endothelial proliferation and deposition of material resembling basement membrane . . ." Thus from the morphologic point of view and as judged from the observations presently available, the development of aminonucleoside nephrosis into a chronic irreversible disease seems to imitate certain forms of pure lipoid nephrosis in children.

Summary

A renal disorder with nephrosis persisting for 1½ years was induced by aminonucleoside injections in rats. During the acute phase of the disorder and for 5 to 10 months the animals manifested a nephrotic syndrome with edema and pronounced proteinuria. Later there was progressive renal failure, hypertension, and death in uremia. Morphologic investigations, including electron microscopy, showed reversible epithelial and endothelial glomerular changes during the acute phase. The most important lesion in the chronic phase was an irregular thickening of the glomerular basement membrane, leading to total hyalinization of the capillary loops.

A comparison has been made between this experimental disorder and the nephrotic syndrome in man, particularly pure lipoid nephrosis in children and membranous glomerulonephritis. It is concluded that from a morphologic point of view there is some similarity between experimental aminonucleoside nephrosis and lipoid nephrosis.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

B = basement membrane
CP = capillary lumen
EN = endothelium
ENN = endothelial nucleus
EP = epithelium
US = urinary space
F = endothelial fenestra
G = Golgi membranes
M = mitochondria
P = epithelial foot processes
V = vacuole
BC = Bowman's capsule
BB = basement membrane, Bowman's capsule

FIG. 1. Phase contrast microscopy; approximately 0.5 μ thickness, methacrylate embedding. Six daily injections of aminonucleoside (very slight proteinuria). The glomerulus fills the capsular space entirely, and there is swelling of endothelium and epithelium with only a few capillary loops containing red cells. Some epithelial cells contain clusters of round dark granules. The proximal convoluted tubules surrounding the glomerulus show focal cytoplasmic swelling with accumulations of dark granules ("protein absorption droplets") at A. × 1,200.

FIG. 2. Chronic aminonucleoside nephrosis with hypertension and renal failure. There is nearly complete hyalinization of glomerular capillary loops; an inflammatory infiltration appears about the glomerulus. The tubule (A) is dilated; its epithelium is low and the lumen contains a hyaline mass. Periodic acid-Schiff stain. × 300.

FIG. 3. Rat glomerulus after 6 injections of aminonucleoside (same animal shown in Fig. 1) before onset of heavy proteinuria. The endothelial cytoplasm is slightly swollen. Foot process organization in the epithelial cells is nearly completely lost, and there is an irregular accumulation of dark granular material in a narrow zone close to the basement membrane. In the epithelial cytoplasm many dark, electron-dense "droplets" of varying size are apparent, and there are also vacuoles and bodies containing moderately electron-dense material (A). × 12,000.
Insert: Another portion of the same glomerulus. Two "myelin figures" (MF) composed of tightly packed parallel membranes are situated in the epithelial cytoplasm. × 31,000.

FIG. 4. Same rat shown in Figure 1. A large, membrane-limited vacuole (A) is situated in the epithelial cytoplasm. It is nearly completely filled with electron-dense, finely granular material. Outside the membrane there is an accumulation of material with the same appearance. × 16,500.

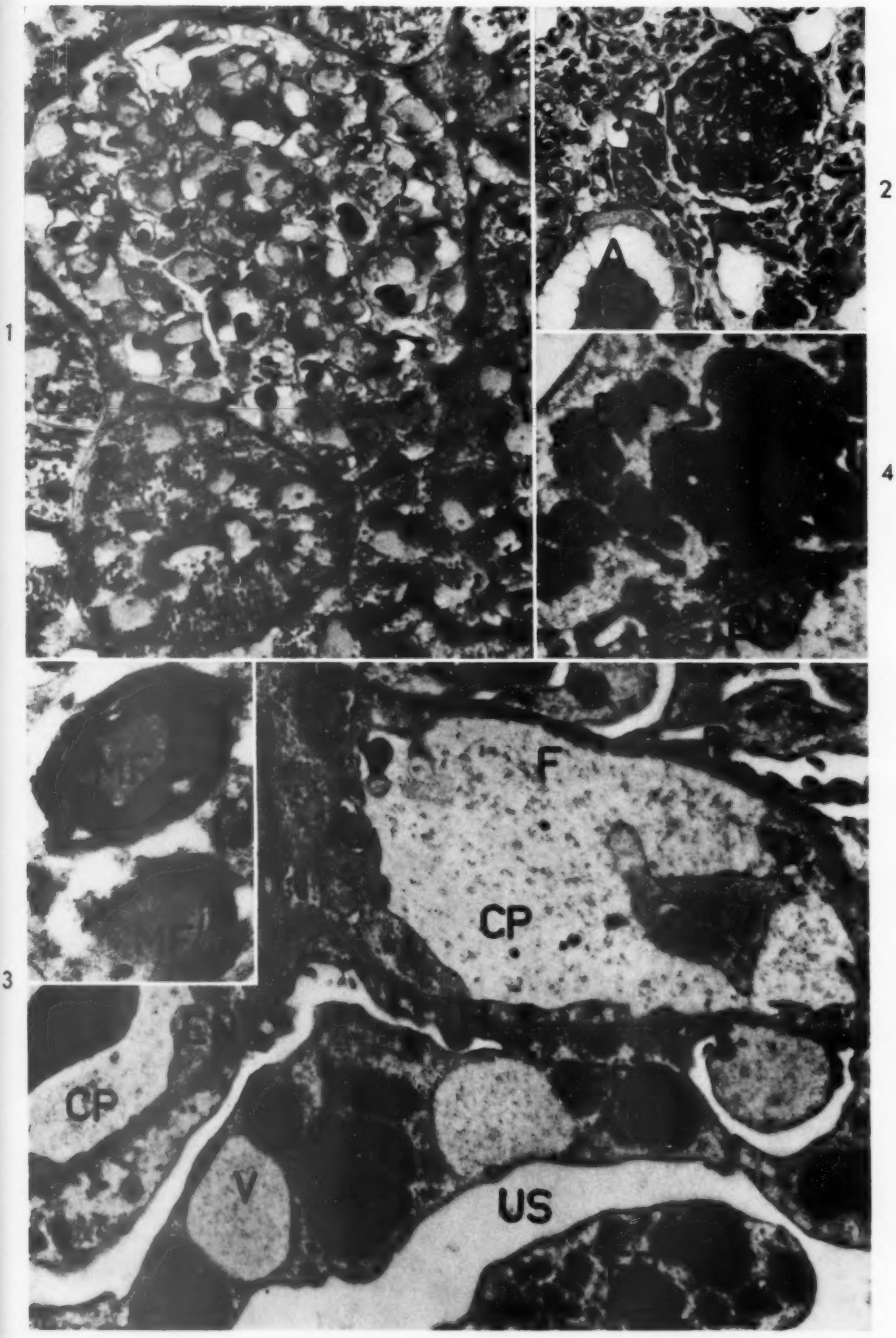
1
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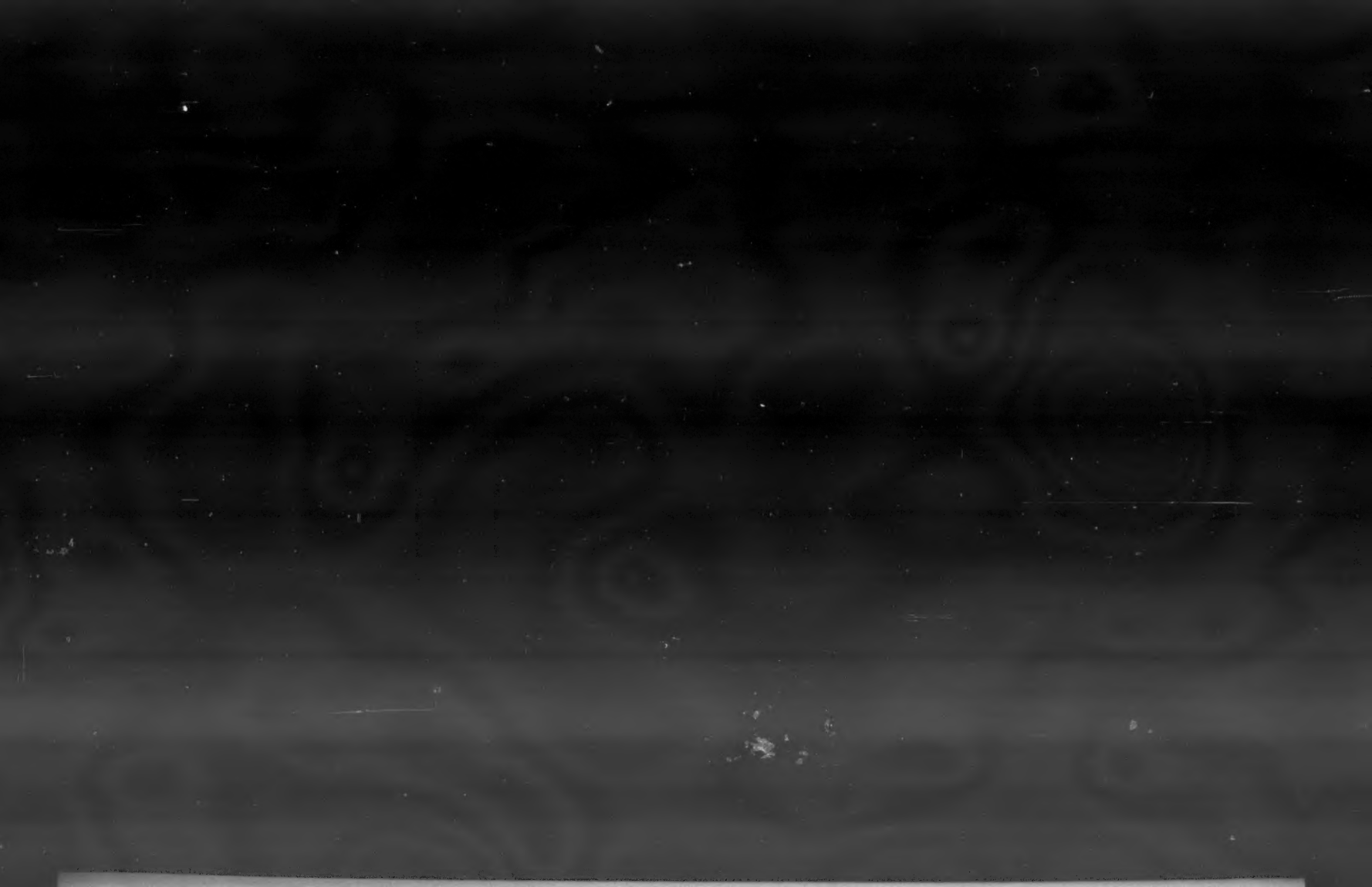
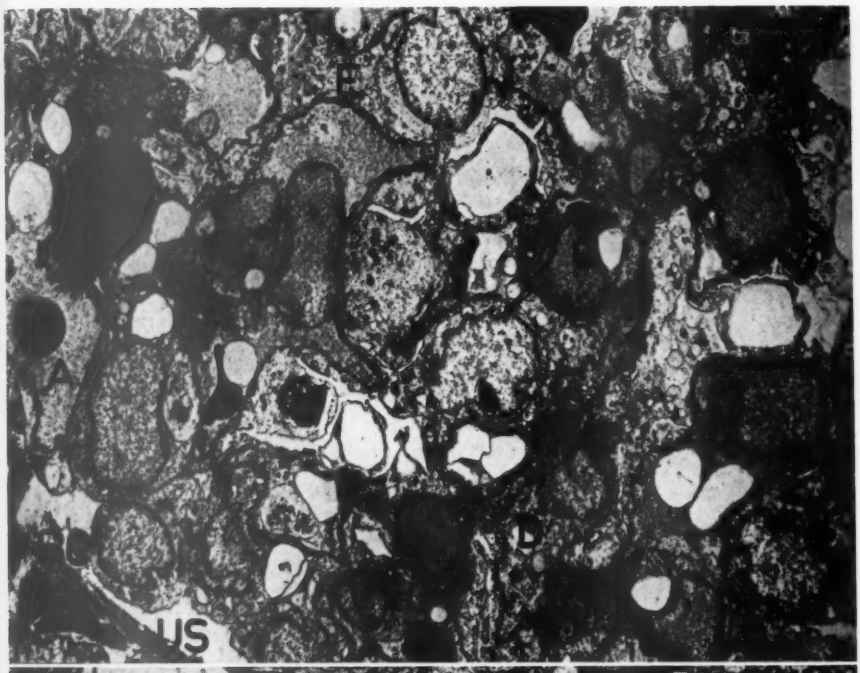
FIG. 5. Acute phase (15 days) in a rat with heavy proteinuria. The number of endothelial cells is increased, and the cytoplasm of both endothelial and epithelial cells is swollen. Some capillary loops are wide and patent (A); most are more or less obliterated (C), but some are closed (D). There is an accumulation of dark material in the epithelial cytoplasm (E) outside the basement membrane. The foot process organization is completely lost in this region as well as in many other areas. Bars of basement membrane-like material appear within an epithelial cell (H). × 4,500.

FIG. 6. Glomerulus from a rat with chronic nephrosis (about 6 months). Heavy proteinuria is unaccompanied by hypertension or renal failure. Many capillary loops are completely obliterated by endothelial proliferation and swelling (A). Epithelial cell changes resemble those seen in the acute stage with swelling of the cytoplasm, which contains many electron-dense "droplets." The basement membrane is wrinkled (C) and irregular, with focal thickening in some portions (D). × 2,800.





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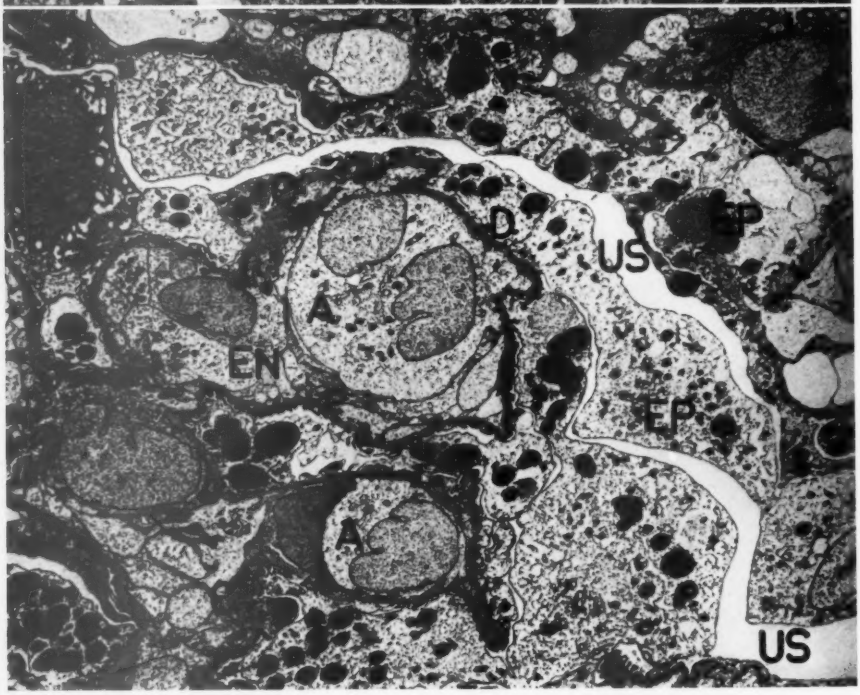

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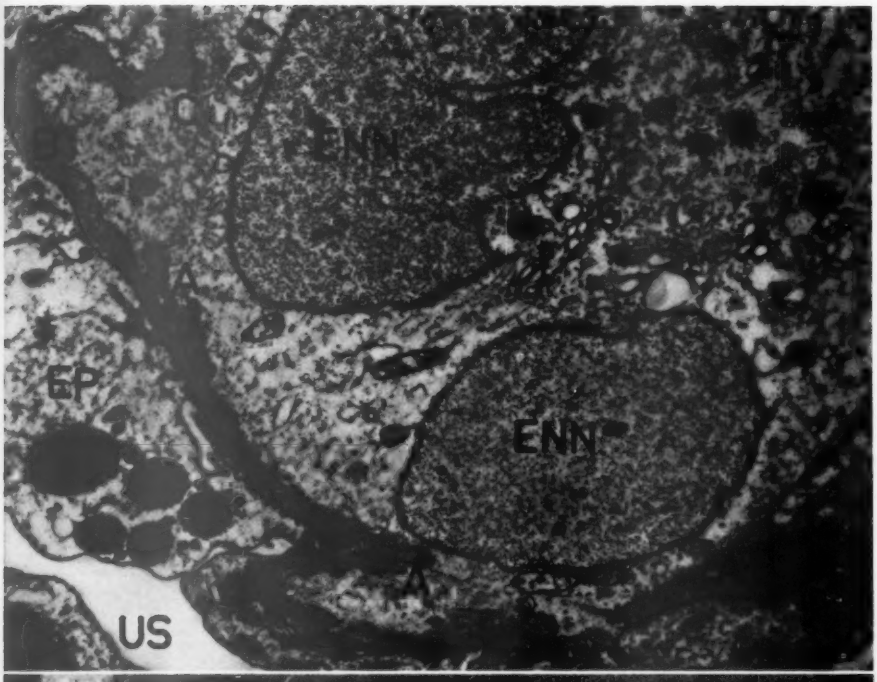
FIG. 7. A higher power view of the area shown in Figure 6. The lumen of the capillary is closed. Outpouchings of the basement membrane (A) extending toward the endothelial side are composed of material having the same density and appearance as the basement membrane proper. Basement membrane outpouchings (C) contain a substance somewhat more granular and loosely arranged than in the basement membrane proper. × 15,500.

FIG. 8. Glomerulus from a rat with chronic nephrosis of about 10 months' duration. There was slight proteinuria and no hypertension or renal failure. The thickness of the basement membrane is varied and never less than 1,600 Å. "Spurs" (A) have formed from the membrane. The content of these outpouchings is of somewhat lighter density than in the rest of the membrane (Al), perhaps corresponding to the lamina rara. The organization of the foot processes is fairly well preserved. × 42,300.



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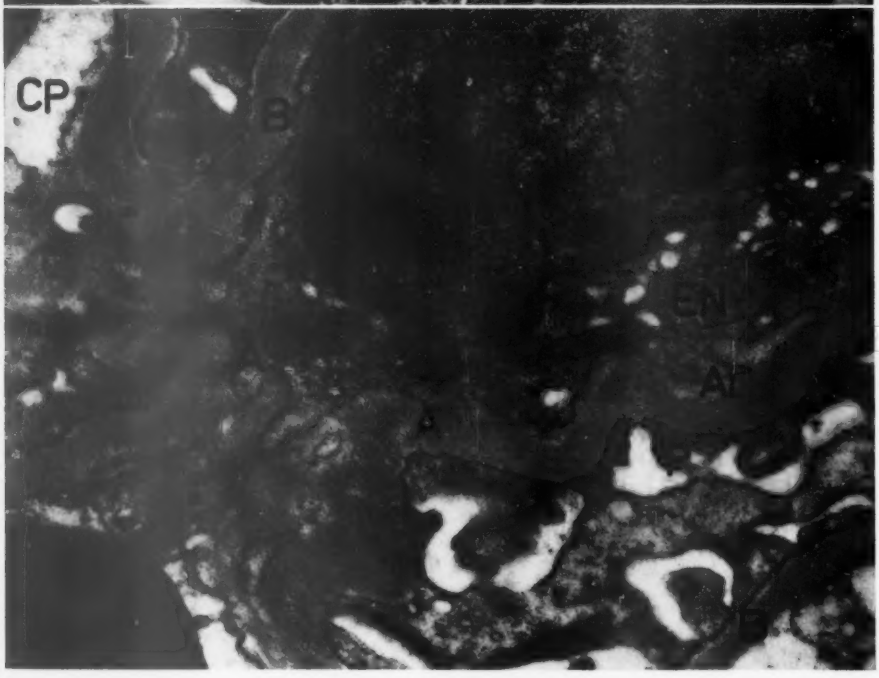

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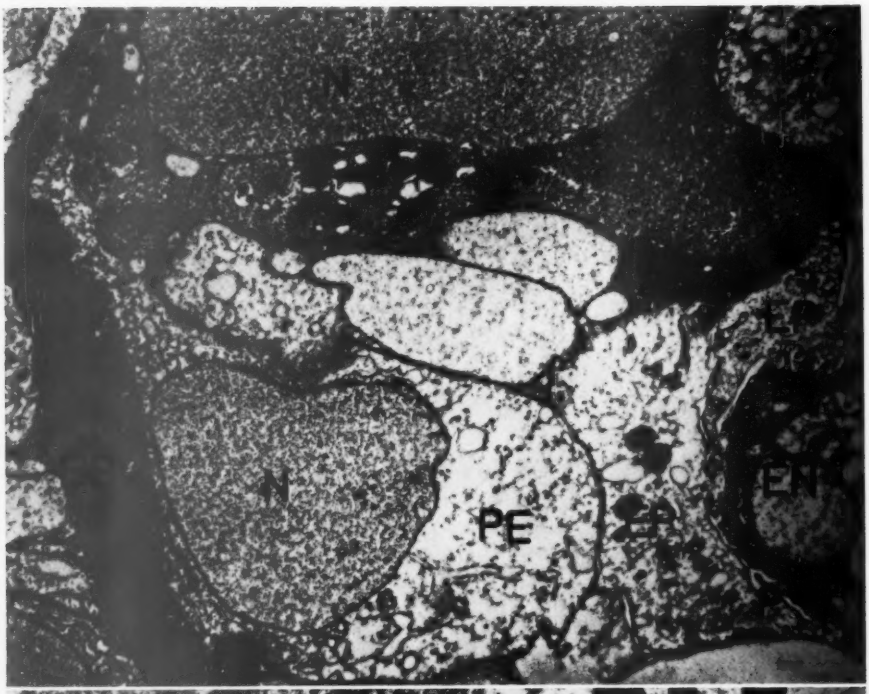
FIG. 9. Peripheral portion of a glomerulus and Bowman's capsule. Rat with chronic nephrosis of 7 months' duration. There was proteinuria but no renal failure or hypertension. Adhesions are evident between the glomerular epithelium and the parietal epithelium of the capsule (PE). The capsular epithelial cells are large and swollen and the basement membrane of the capsule is thickened and lamellated. Dark substance (A) has accumulated between the glomerular and capsular epithelium. N indicates the nucleus of a capsular epithelial cell. × 7,800.

FIG. 10. Glomerulus and Bowman's capsule from a rat with chronic nephrosis in the terminal stage (renal failure, hypertension). The endothelial cytoplasm is nearly completely replaced by a mass with the same (or somewhat darker) density as the basement membrane. The epithelium is unaffected by this substance but shows flattening and coalescence of foot processes, cytoplasmic edema, and many electron dense "droplets." In the upper part of the figure there is an adhesion between a glomerular (EP) and a capsular (PE) cell. Nucleus of a capsular cell (N). × 16,000.

Insert: Crescent formation in a glomerulus. The basement membrane of Bowman's capsule is thickened and lamellated. The lamellas are split into a network with a finely granular, rather loosely arranged substance in its meshes. The material from the enormously thickened capsular basement membrane is in close contact with epithelial cells and the basement membrane of glomerular capillary loops. × 6,500.



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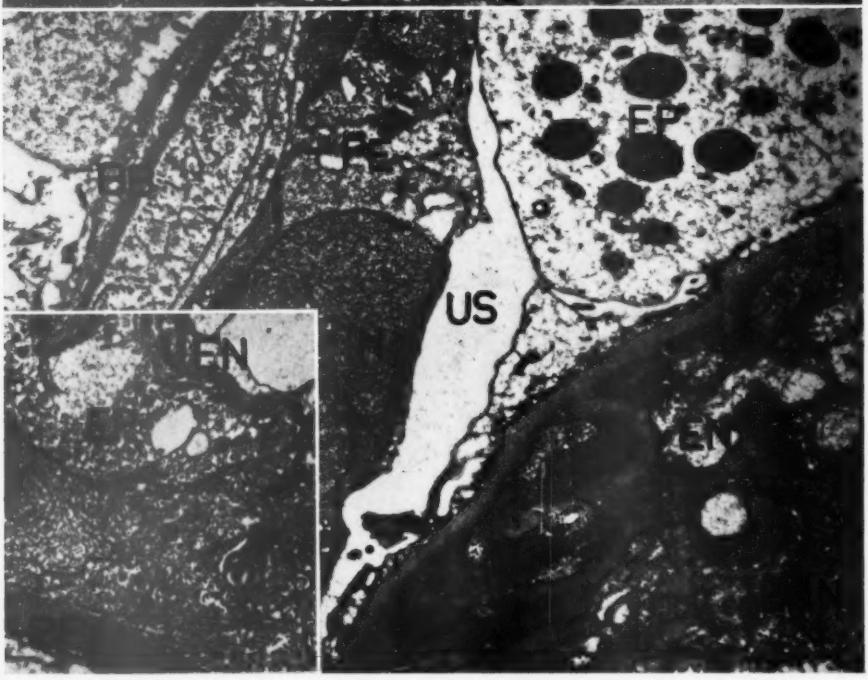

10

EFFECT OF HYPERTENSION ON VASCULAR AND OTHER LESIONS OF SERUM SICKNESS

EDWIN R. FISHER, M.D.,* AND JACK BARK, M.D.†

From the Departments of Pathology, University of Pittsburgh and Veterans Administration Hospital, Pittsburgh, Pa.

There is abundant experimental and clinical evidence to indicate that hypertension may induce the development of widely distributed vascular lesions. In some experimental situations,[1–8] as well as in patients succumbing to malignant hypertension,[9,10] such lesions have exhibited a similarity to the vascular alterations characteristic of polyarteritis nodosa in man. Indeed, as first suggested by Meyer in 1878,[11] some have ascribed a significant role to hypertension in the pathogenesis of this vascular disorder, at least in certain instances.[2,3,12] Although hypertension may be absent in patients with polyarteritis, nevertheless the relationship between the occurrence of lesions in the pulmonary arteries and the existence of pulmonary hypertension does appear direct.[13] Similarly, a close resemblance between the lesions of drug sensitivity and serum sickness in man [14,15] and the rabbit [16–20] and polyarteritis has been commented upon by a number of investigators. However, that there are significant histologic differences between the vascular lesions resulting from hypertension and the injection of foreign protein has been recently emphasized by Campbell and Santos-Buch [21] who compared the histologic effects of hypertension produced by unilateral perinephritis and hypersensitivity following the injection of horse serum in rabbits.

Because of the possible etiologic significance of hypertension in the development of the vascular lesions in polyarteritis as well as its effect on vascular lesions in general, it was considered worth while to explore the effect of the hypertensive state on the vascular lesions induced in rabbits by the administration of foreign protein. This report is concerned with the results of such a study dealing with the effect of hypertension on the acute lesions of hypersensitivity as well as those in the healed stage.

MATERIAL AND METHODS

Eighty-three adult white male and female rabbits, weighing approximately 2.5 kg., were utilized in the following experiments. Body weights were recorded prior to and at 2-week intervals during the experimental periods. Animals were maintained on a

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* Senior Research Fellow, United States Public Health Service.

† Formerly Senior Medical Student, University of Pittsburgh.

stock laboratory ration and water *ad libitum*. Blood was obtained prior to sacrifice for determination of the blood urea nitrogen (BUN) by the Kjeldahl technique.

Group I. Induction of Hypertension

The induction of hypertension was attempted in 15 rabbits by the method of Page, Salmoiraghi and McCubbin [22] except that both unilateral nephrectomy and cellophane enclosure of the contralateral kidney were performed in one stage. Ten exhibited an average elevation of blood pressure greater than 15 mm. of Hg throughout the experimental period and were considered as hypertensive. Hypertension was first noted 7 to 10 days following operation. Animals in this group were sacrificed 30 to 50 days (average 43 days) following the onset of hypertension or after operation.

Group II. Production of Lesions of Acute Hypersensitivity

Eighteen rabbits received a single intravenous injection of 250 mg. per kg. of body weight of bovine serum albumin (BSA) (Armour & Company), which was trace-labeled with I^{131} (IBSA) according to the method of Talmage, Dixon, Bukantz and Dammin.[23] Animals were bled 5 minutes after injection and every 1 to 3 days starting on the third day following injection. Total IBSA activity was determined in aliquots of serums and converted to per cent IBSA of the initial 5-minute sample. Twelve animals exhibited curves characteristic of immune elimination of antigen between 13 to 16 days following injection.[20,24] These were sacrificed on the day of antigen elimination. The remainder of the group were empirically sacrificed on day 17.

Group III. Healed Hypersensitivity

Twelve rabbits were treated identically to those described in group II except that they were all sacrificed 28 days following injection of IBSA. Eight had demonstrated a curve indicative of immune elimination of antigen.

Group IV. Effect of Hypertension on Lesions of Acute Hypersensitivity

The induction of hypertension was attempted in 22 rabbits. All received an injection of IBSA as noted in group II 32 days after operation. Twelve remained hypertensive throughout the experimental period and exhibited immune elimination of antigen 13 to 16 days following injection and were sacrificed at corresponding periods. Five hypertensive rabbits failed to exhibit immune elimination of antigen and were sacrificed 17 days following IBSA injection. In the remaining 5, hypertension failed to develop or persist to the time of IBSA injection. Three of these exhibited curves characteristic of immune antigen elimination.

Group V. Effect of Hypertension on Healed Lesions of Hypersensitivity

Sixteen rabbits in which hypertension had been successfully induced for approximately 15 days received an injection of IBSA as noted in group IV. Ten exhibited immune elimination of antigen 13 to 16 days following injection. All animals were sacrificed 29 days following injection of IBSA except one which died 22 days following injection.

Estimation of Blood Pressure

Blood pressure determinations were performed by the ear capsule technique of Grant and Rothschild.[25] All readings were obtained by the same individual on animals in the same isolated room. A control blood pressure was obtained for each rabbit by calculating the average of 3 consecutive daily determinations. Blood pressure was determined every other day for 10 days following operation and every week thereafter.

Anatomic and Histologic Studies

At the time of sacrifice or death of the animals (one of group V, *vide supra*), the heart was cleaned of pericardial fat and adventitious tissue, and weighed. Comparable

blocks of heart, lung, aorta, pancreas, spleen, kidney, gonad, skeletal muscle, stomach, small intestine, colon, thyroid and adrenal from all animals were fixed in 10 per cent formalin and processed and embedded in paraffin in the usual manner. Sections were stained with hematoxylin and eosin, and by the Verhoeff-van Gieson method in selected instances.

The severity of lesions encountered was subjectively graded 1 to 4^+. An arbitrary index of the degree of the vascular alterations encountered in each animal was expressed as the quotient of the sum of the grades of severity of lesions encountered in each organ divided by the number of organs affected.

Results

Two rabbits in group II (normotensive, acute hypersensitivity) and a similar number in group IV (hypertensive, acute hypersensitivity) that did not exhibit immune elimination of antigen were found to have lesions comparable to other members of group II (*vide infra*) when empirically sacrificed at 17 days. However, only those animals receiving IBSA, demonstrating characteristic curves of this phenomenon, were included in the final tabulation of results. This practice afforded a common "end point" for the comparison of results obtained in the various experimental groups receiving IBSA.

General Effect of Cellophane Perinephritis and Injections of Foreign Protein (IBSA)

No significant changes in body weight were observed in any of the experimental groups (Table I). Animals subjected to cellophane perinephritis and contralateral nephrectomy exhibited less weight gain during the experimental period than untreated, unoperated control rabbits during a comparable period of observation. This effect did not appear to be related to the successful induction of hypertension in these animals. Moderate but comparable elevations of BUN were noted in all animals subjected to operation as well as those that received injections of IBSA and were sacrificed at the time of its immune elimination (groups II and IV). The administration of IBSA failed to influence the blood pressure in either normotensive or hypertensive animals.

Comparison of Lesions in Hypertensive Rabbits and Those Receiving IBSA

Aside from cardiac hypertrophy, the lesions observed in animals with successfully induced hypertension (group I) were almost exclusively limited to arteries of distributing and smaller caliber. As indicated in Table II, these were most frequently observed in the gastrointestinal tract and liver, and only rarely in a branch of the coronary arteries (Fig. 1). The vascular alteration was characterized by either medial hypertrophy with moderate lumen narrowing or mural hyalinization. Necro-

TABLE I

OBSERVATIONS IN NORMOTENSIVE AND HYPERTENSIVE RABBITS WITH AND WITHOUT HYPERSENSITIVITY (BSA)

Group		No. of animals	Weight (kg.)			Blood pressure (mm. of Hg)			Duration of hypertension (days)		Days following BSA injection	Antigen elim. (day)	BUN † (mg. %)
			Begin	End	Difference	Begin	End *	Difference	Total	Before BSA			
I	Hypertension	10	2.3	2.5	+0.2	82	114	+32	43				30
II	BSA, acute, normotensive	12	3.0	3.0	0	84	81	− 3			13—15	13—15	28
III	BSA, healed, normotensive	8	2.9	2.9	0	74	78	+ 4			28	13—16	21
IV	Hypertension and BSA, acute	12	3.0	3.0	0	82	128	+46	45	32	13—16	13—16	35
V	Hypertension and BSA, healed	10	2.4	2.6	+0.2	84	113	+29	44	15	28‡	12—16	38

* Average from onset of hypertension.
† Normal range, 10 to 20 mg. %.
‡ One died at 22 days.

TABLE II

LESIONS IN NORMOTENSIVE AND HYPERTENSIVE RABBITS WITH AND WITHOUT HYPERSENSITIVITY (BSA)*

Group		Glomerulo-nephritis	Heart wt. (gm.)	Valvulitis	Spleen (granuloma)	Arteritis					Index of severity of arteritis
						Kidney	Heart	G.I.	Liver	Misc.	
I	Hypertension	0	9.1	0	0	0	10/1	30/2.5	30/2	10/2	1.9†
II	BSA, acute, normotensive	50/1.2	7.1	50/1	17/2	17/2	25/1.5	0	0	0	1.7
III	BSA, healed, normotensive	0	6.9	13/1	0	0	0	0	0	0	0
IV	Hypertension and BSA, acute	75/2.1	8.6	75/1.8	17/1	8/3	75/1.8	58/2.7	17/2.5	42/1.8	2.4
V	Hypertension and BSA, healed	0	9.0	0	0	0	20/2	10/2	10/3	0	2.3

* Lesions represented as per cent incidence/grade of severity.
† Lesions, hyaline or proliferative; arteritis in only one instance of group I.

tizing arteritis with fibrinoid change of the medial coat and a moderate heterophil and lymphocyte cellular infiltration in all vessel coats was noted in only one instance. Examination of animals subjected to perinephritis in which hypertension failed to develop did not disclose any significant vascular or other alteration.

On the other hand, 50 per cent of normotensive rabbits examined following the immune elimination of injected IBSA (group II) disclosed the presence of proliferative glomerulonephritis and a valvulitis (Fig. 2). The latter was characterized by endothelial swelling and hyperplasia, subendothelial edema and infiltration of the cardiac valves with varying numbers of inflammatory cells. A granulomatous reaction was observed in the white pulp of the spleen in only one animal of this group. Arteritis of medium and small arteries, principally the coronary and renal arteries, was also evident (Fig. 3). The vascular lesions varied in intensity in a manner similar to that noted above as occurring within the heart valves but, in addition, not infrequently exhibited fibrinoid necrosis. Except for the presence of mild valvulitis encountered in one animal, rabbits of group III examined two weeks following the immune elimination of antigen failed to reveal any significant vascular or other alteration.

Effect of Hypertension on Lesions of Acute and Healed Hypersensitivity

As noted in Table II, the administration of IBSA to hypertensive rabbits (group IV) resulted in a marked increase in the incidence of glomerulonephritis (Fig. 4), valvulitis (Fig. 5) and arteritis (Fig. 6) when these animals were examined at the time of immune elimination of antigen. In addition, a marked increase of vasculitis was observed in sites more frequently affected by uncomplicated hypertension, viz., gastrointestinal tract (Fig. 7) and liver. Also, arteritis was observed in the gonad, thyroid, and lung in a substantial number of these animals although such lesions were not observed in either hypertensive (group I) or hypersensitivity (group II) controls. Although there were no qualitative differences observed in the lesions of hypersensitivity noted in hypertensive animals as compared to similarly treated normotensive controls, the intensity of the nephritis, vascular lesions and arteritis was more severe as reflected by the results of the arbitrary grading technique employed in this study for the estimation of degree of alteration at these sites (Table II). This effect did not appear to be directly related to the degree of elevation of blood pressure encountered.

It is also apparent from Table II that the hypertensive state had no effect on the healing process in the lesions of hypersensitivity. Rabbits of this group (IV) as well as members which did not exhibit immune elimination of antigen displayed lesions (Figs. 8 and 9) qualitatively and quantitatively similar to those in the hypertensive controls (group I).

Discussion

The results of this study have indicated that the hypertensive state increased the incidence as well as accentuated the vascular, valvular and glomerulonephritic lesions of acute serum sickness induced by the administration of BSA. This effect is also reflected by a comparison of our results concerning the general incidence of acute lesions in hypertensive BSA-treated rabbits and those appearing in the apparently normotensive BSA-treated rabbits reported by Germuth[19] and Weigle and Dixon[24] (Table III). Analysis indicates that except for a slightly lower incidence

Table III

AVERAGE INCIDENCE OF HYPERSENSITIVITY LESIONS AT TIME OF ANTIGEN ELIMINATION IN NORMOTENSIVE AND HYPERTENSIVE RABBITS

Investigator	No. of animals	Blood pressure	Glomerulonephritis (%)	Valvulitis (%)	Arteritis (%)
Germuth [19]	9	Normotensive	89	44	56
Weigle and Dixon [24]	19	Normotensive *	78	53	47
Fisher and Bark	12	Normotensive	50	50	41
Fisher and Bark	12	Hypertensive	75	75	83

* Presumably normotensive; no mention of blood pressure.

of glomerulonephritis in the normotensive BSA-treated animals, similar values were obtained for other lesions of acute hypersensitivity, and the latter, particularly arteritis, were increased in animals with hypertension. It appears worth while to note that the incidence of coronary arteritis was profoundly influenced by the hypertensive state (Table II). In addition, hypertensive rabbits receiving BSA frequently exhibited vascular lesions in the gastrointestinal tract, liver and such miscellaneous sites as the thyroid and gonad, a distribution unlike that of the lesions in acute hypersensitivity but similar to that observed in untreated hypertensive controls. This information raises the query whether hypersensitivity might not have augmented the lesions ascribed to hypertension. Although we cannot unequivocally exclude this possibility, certain considerations tend to militate against it. The lesions encountered in the hypertensive rabbits exhibiting hypersensitivity appeared more intense than those observed in normotensive rabbits treated with BSA. Nevertheless they were qualitatively similar to those of the latter group, being characterized by varying degrees of vascular necrosis and fibrinoid change with a conspicuous inflammatory component. On the other hand, the lesions observed in the hypertensive controls in this and previous studies in which

this experimental model was utilized, were, except on rare occasions, predominantly noninflammatory, being characterized by hyalinization of vascular coats or medial hypertrophy. It might be noted that our findings in this regard are in general agreement with those of Campbell and Santos-Buch [21] who observed a similar difference in the distribution pattern and histologic appearance of the lesions produced by hypertension induced by perinephritis and serum sickness resulting from the administration of foreign protein. Further, these authors rarely noted the occurrence of a necrotizing vascular lesion after 22 days of hypertension. The duration of hypertension in the studies reported herein was 40 or more days. Since cardiac valvular lesions as well as glomerulonephritis have been found only in rabbits receiving BSA, one would be compelled to conclude that the vascular lesions observed in the gastrointestinal tract were pathogenetically different from those noted in other sites. On the other hand, as indicated above, the qualitative similarity, although not wholly corroborative, does lend some support to their pathogenetic similarity. Lastly, if hypersensitivity primarily affected the lesions of hypertension, which at the time of administration of BSA should have been histologically "healed," one might expect to observe some evidence of chronicity in the lesions. Foci of necrosis have been observed in "healed" lesions of polyarteritis nodosa in man; these have been considered strongly suggestive of such an event.[26]

The mechanism responsible for the augmentation of the acute lesions of hypersensitivity in hypertensive rabbits is not clearly evident. There is some evidence to warrant the consideration that this effect is the result of the hypertension *per se*. No significant differences in the degree of nitrogen retention or change in body weight could be detected which might be construed to play a role in accounting for the divergent observations. More direct support for the primacy of the role of hypertension may be obtained from the animals exhibiting immune elimination of antigen, in which hypertension failed of induction or failed to persist throughout the experimental period. The incidence and severity of lesions in these were comparable to those encountered in normotensive BSA-treated rabbits. Since no significant fluctuations in blood pressure were noted in hypertensive or normotensive rabbits subjected to BSA injection, it seems highly unlikely that the effects observed might be due to blood pressure variations. In this regard, it is of interest that no direct relationship could be discerned between the degree of blood pressure elevation among the individual hypertensive BSA-treated rabbits and the severity of vascular lesions. Whether hypertension affects the nature of antigen-antibody complexes probably playing a role in the pathogenesis of serum sickness [20,27] or renders vessels more susceptible to their action remains to be demonstrated. In regard to the latter, it is note-

worthy that hypertension clearly accentuates the development and severity of another vascular disease, notably cholesterol atherosclerosis,[28] and that this effect may supersede such inhibitory or protective influences as diabetes[29] or the administration of cortisone.[30] The effect of hypertension in this regard has been considered attributable to its damaging effect on vessels.

Although the acute lesions of hypersensitivity were augmented by hypertension, this modality had no effect on the so-called healed lesions (group IV) in which vascular and other lesions were only exceptionally apparent in normotensive animals (group III). Qualitatively and quantitatively, the lesions in such animals resembled those encountered in the hypertensive controls. This information indicates that hypertension does not interfere with the healing process encountered in the vasculitis of hypersensitivity, despite the greater intensity of these lesions during their acute stage than observed in similarly treated normotensive rabbits. Moreover, hypertension was incapable of prolonging the duration of the lesions of hypersensitivity or producing vascular or other sequels.

This information also signifies the primacy of more prolonged antigenic stimulation or factors other than hypertension in the production of persistent vascular lesions considered to be immuno-allergic in nature. Although analogies between these experiments and the possible relationship of hypertension and polyarteritis or other forms of vasculitis in man do not appear warranted, it is well recognized that the lesions of polyarteritis nodosa may exhibit healing despite the presence of hypertension. Indeed, this characteristic represents one of the diagnostic criteria of this vascular disorder.

Summary

The successful induction of hypertension resulted in an increased incidence and severity of the renal, cardiac and vascular lesions, particularly those of the coronary arteries, in rabbits with serum sickness, as compared to normotensive controls. The hypertensive rabbits with serum sickness also displayed a high incidence of arteritis of the gastrointestinal tract, liver, and other sites more characteristic of the distribution resulting from uncomplicated hypertension. However, in the latter instance the vascular alterations were notably proliferative or hyaline rather than inflammatory as in the case of animals receiving foreign protein. This effect of the hypertensive state appears to be related to the hypertension *per se*. Its exact pathogenetic mechanism in this regard, however, remains to be elucidated. Attention is directed to the recognized damaging effect of hypertension on vessels, which may render the latter more susceptible to the effects of serum sickness.

On the other hand, hypertension failed to influence the resolution

of the lesions of serum sickness in rabbits sacrificed 1 to 2 weeks following the immune elimination of antigen. It is concluded that hypertension exerts little influence on the lesions of serum sickness, which in some respects resemble those of polyarteritis nodosa in man, except during their acute phase.

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[*Illustrations follow*]

Legends for Figures

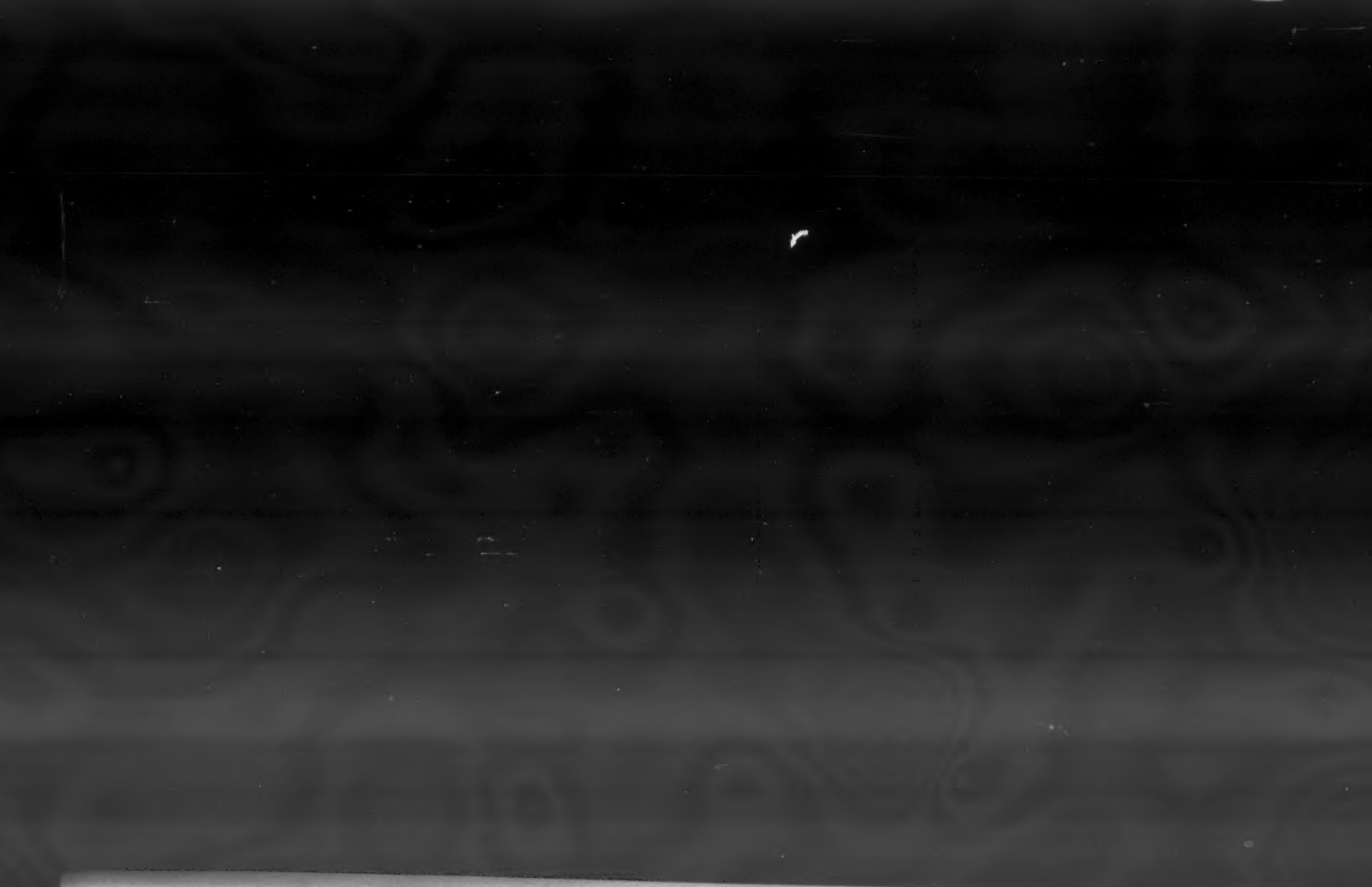
Photomicrographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. A branch of the hepatic artery from a hypertensive control rabbit (group I) exhibiting marked medial thickening. × 100.

Fig. 2. A representative degree of valvulitis in a normotensive rabbit which received an injection of bovine serum albumin (BSA). Endothelial proliferation, edema and scant inflammatory infiltrate are evident. The reaction is less severe than that noted in Figure 5. × 100.

Fig. 3. A mild degree of coronary arteritis in a normotensive rabbit receiving an injection of BSA. Compare with the alteration depicted in Figure 6. × 100.

Fig. 4. Proliferative glomerulonephritis of moderate degree in a wrapped kidney from a hypertensive rabbit that received BSA and was sacrificed at the time of immune elimination of antigen. × 100.



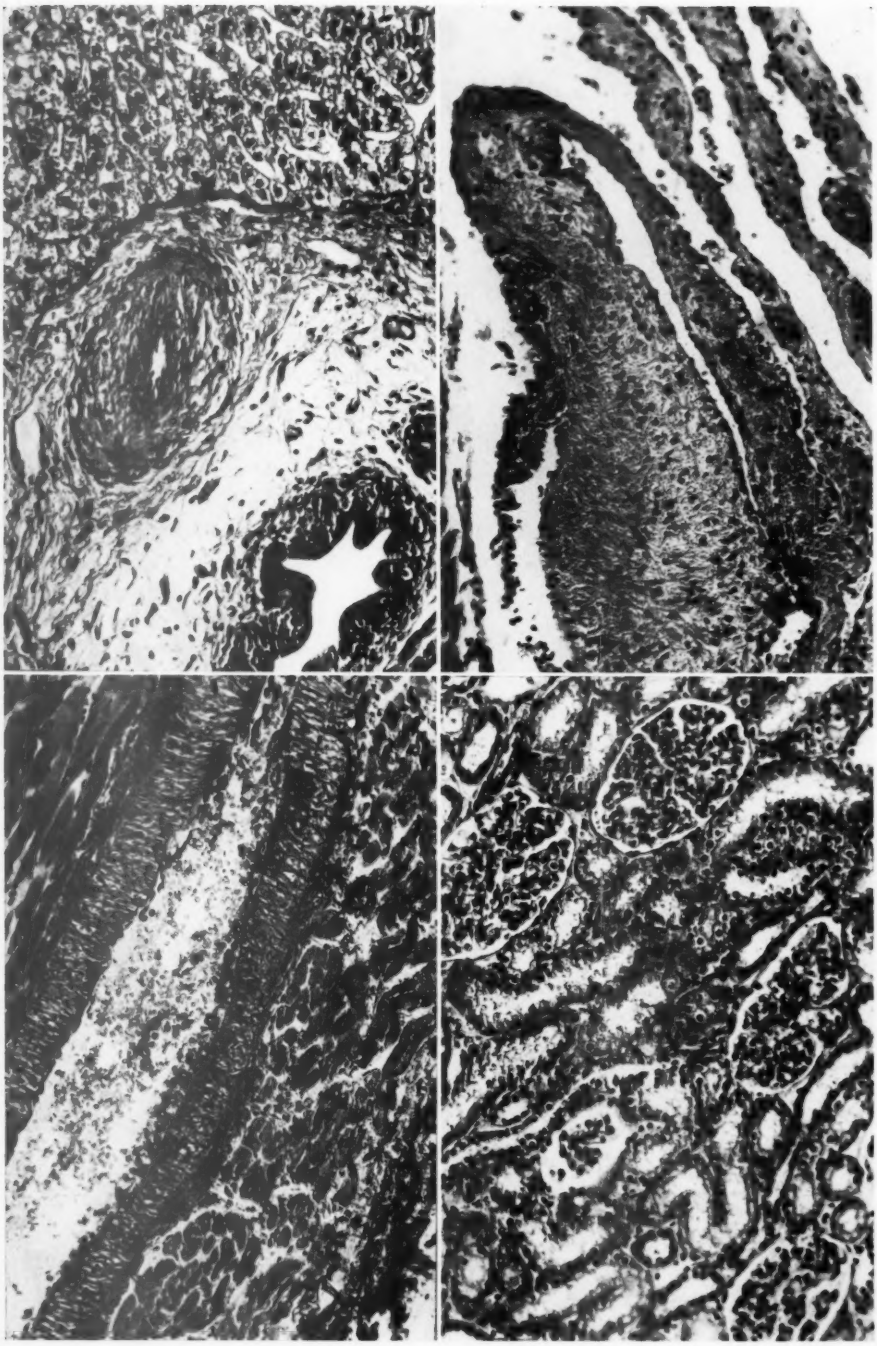


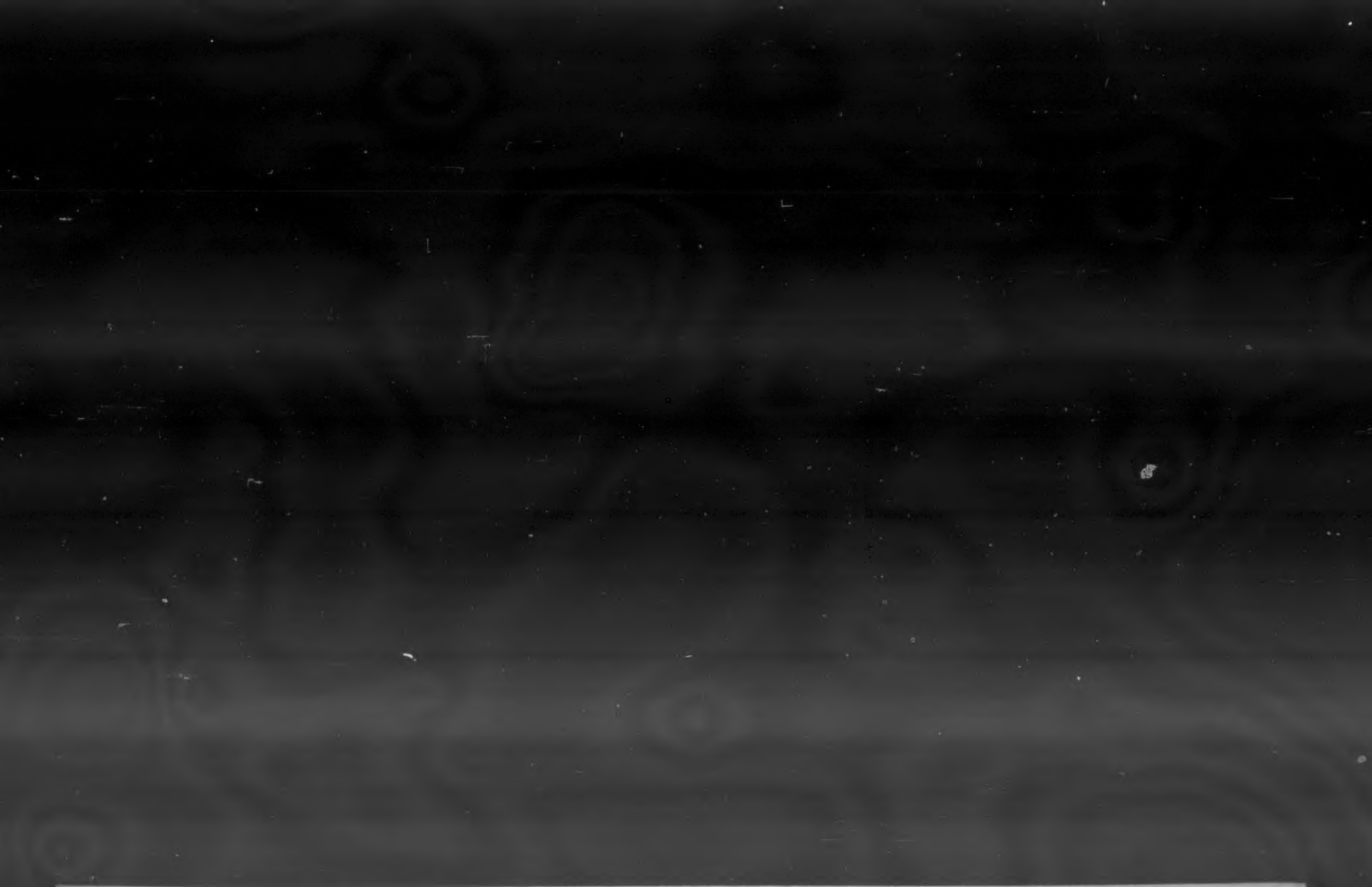
FIG. 5. Severe valvulitis is observed in a hypertensive rabbit receiving BSA (group IV) and sacrificed at the time of immune elimination of antigen. × 100.

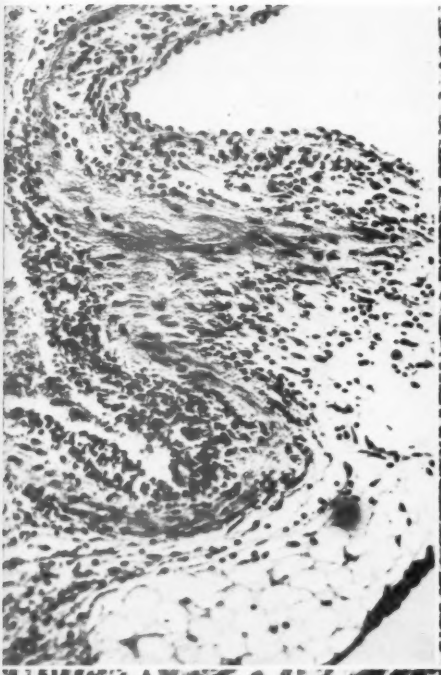
FIG. 6. Severe coronary arteritis is observed in a hypertensive rabbit receiving BSA (group IV) and sacrificed at the time of immune elimination of antigen. × 100.

FIG. 7. Severe arteritis appears in the submucosal vessels of the stomach in a hypertensive rabbit receiving BSA and sacrificed at the time of immune elimination of antigen. × 100.

FIG. 8. A hyalinized submucosal artery in the small intestine of a hypertensive rabbit that received BSA but was sacrificed several weeks after immune elimination of antigen (group V). × 100.

FIG. 9. Medial hypertrophy is apparent in a branch of the coronary artery from a hypertensive rabbit sacrificed after immune elimination of antigen (group V). The lesion is qualitatively similar to that in the hypertensive control depicted in Figure 1. × 100.





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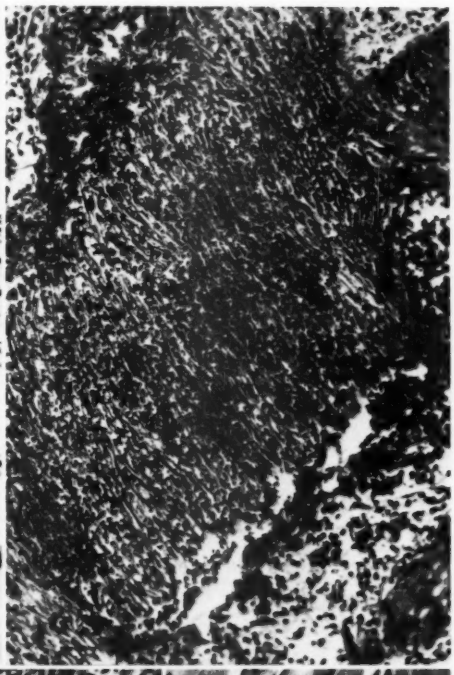

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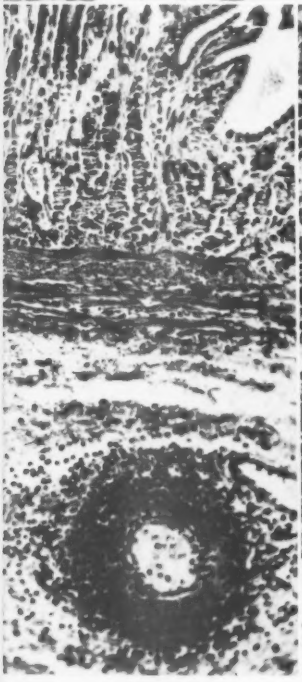

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THE RESPONSE OF THE GERM-FREE GUINEA PIG TO ORAL BACTERIAL CHALLENGE WITH ESCHERICHIA COLI AND SHIGELLA FLEXNERI

With Special Reference to Lymphatic Tissue and the Intestinal Tract

Helmuth Sprinz, Col., M.C., U.S.A.; Donald W. Kundel, Capt., M.C., U.S.A.; Gustave J. Dammin, M.D.; Richard E. Horowitz, Capt., M.C., A.U.S.*; Herman Schneider, M.S., and Samuel B. Formal, Ph.D.

From the Departments of Experimental Pathology, Applied Immunology, and Germ-free Research, Walter Reed Army Institute of Research, Washington, D.C., and the Department of Pathology, Harvard Medical School, Boston, Mass.

The germ-free animal represents an exceptional experimental model[1] for the study of many host-parasite relationships. The availability of such animals has opened the road to investigation of the role of various micro-organisms in the digestive tract, of the effects of microbial interaction in the intestine, and of the changes produced by the introduction of organisms into an alimentary tract previously unchallenged by living bacteria. These implications were recognized by the pioneers in this field,[2] but the objectives they so clearly stated awaited the technical perfection of rearing germ-free animals, only recently available.

The present study is concerned with the reaction of the germ-free guinea pig to intestinal micro-organisms. The conventional guinea pig, unless previously modified by starvation or the administration of carbon tetrachloride, is resistant to oral challenge with *Shigella flexneri*.[3] Germ-free guinea pigs similarly challenged die. In contrast, they survive contamination with a strain of *Escherichia coli* and are then resistant to subsequent challenge with *S. flexneri*,[4] behaving in this regard like conventional animals. The purpose of this report is to record the histologic alterations occurring in the intestine, the mesenteric lymphoid tissue and the adrenal gland of germ-free guinea pigs contaminated with *E. coli* and/or *S. flexneri*. Morphologic alterations of other tissues are not sufficiently pertinent to the theme of our paper to warrant description.

Material and Methods

Detailed information on the methodology employed in raising germ-free guinea pigs, on the strains of bacteria used, and on the technique of oral bacterial challenge has been reported in another paper.[4]

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* Present address: The Mount Sinai Hospital, New York, N.Y.

The following groups of Hartley strain animals were utilized in this study:

1. Guinea pigs (CONV) raised in the conventional manner in a pathogen-free but not germ-free environment and unmodified by any but natural challenges. These were 42 days old.

2. Germ-free guinea pigs (NGF), not exposed to bacteria. These also were 42 days old.

3. Guinea pigs (GFC), raised initially as in group 2, but then exposed to *E. coli*, strain HS, as a monocontaminant for 6 days prior to sacrifice. These were 48 days old.

4. Guinea pigs (GF-C&S) reared germ-free for 42 days, then exposed to *E. coli* as in group 3 (GF-C). Seven to 21 days later they were challenged with *S. flexneri* 2a, strain 2457, and sacrificed 7 to 22 days thereafter, at an age of 56 to 84 days.

5. Guinea pigs (GF-SI) raised germ-free and exposed at the age of 42 days to *S. flexneri* only.

Tissues for histologic studies were processed in a conventional fashion except that spools of opened small and large intestine were prepared by gently rolling them over a forceps.

Results

The immunologic and bacteriologic aspects of this investigation have been reported elsewhere.[4]

The intestinal mucosa of CONV guinea pigs was characterized by a well-developed lymphoreticular stroma containing numerous plasma cells and eosinophils, and by deep crypt glands lined predominantly by chief cells (Fig. 1). In the cecum the mucosa was nonvillous, and goblet cells were largely restricted to the base of the crypts.

In the NGF group the intestinal mucosa resembled the gut at a prenatal stage of development; the lamina propria constituted only a fraction of that seen in CONV animals and contained but few histiocytes, lymphocytes and occasional leukocytes (Fig. 2). While the over-all height of the mucosa was the same as in CONV animals, the crypt glands were shallow. In contrast, however, the slender intestinal villi were tall. Goblet cells similar in number to those in CONV animals were encountered on the villi. The epithelial lining was remarkably uniform even up to the tips of the villi, and showed a distinct brush border. In the ileum, crypt glands were lined almost entirely by markedly distended goblet cells. Mitotic figures in the crypts occurred with equal frequency in both NGF and CONV groups.

Focally the mucosa of the cecum in NGF animals retained a villous pattern. The mucus content of goblet cells was highly variable, in contrast to that of CONV animals. In some areas, mucus had been discharged while in other regions, the crypts were lined with markedly distended goblet cells (Fig. 7).

The small intestine in the GF-C group exhibited features intermediate between those observed in NGF and CONV animals (Fig. 3). The villi were slightly thickened. The crypt glands were shallow and lined by a greater proportion of chief cells; goblet cells were reduced in number. The tunica propria contained a moderate number of macro-

phages; in addition there were lymphocytes, occasional neutrophils, eosinophils and rare plasma cells. An eosinophilic precipitate appeared in the distal interstices of the villi. The epithelium at the tips of the villi showed focal degenerative changes similar to those seen in CONV animals.

The small intestine mucosa in GF-C&S animals approached that of CONV guinea pigs. There were variable elongation of crypt glands, increased cellularity in the tunica propria and reduction in numbers of goblet cells and of the mucus content in individual goblet cells. Similar alterations were observed in the mucosa of the large intestine. Thus, the gut of the GF-C&S group differed slightly from that of the GF-C animals but significantly from that of the NGF group.

The GF-SI group exhibited an acute ulcerative enterotyphlitis, most marked in the lower ileum and cecum (Figs. 5 and 6). The ileal villi were shortened, blunted and fused; in some instances there was obliteration of the villous architecture and a flattening of the mucosal surface (Fig. 4). Crypts were greatly elongated and lined by densely packed, small, hyperchromatic chief cells. In both the ileum and cecum, goblet cells were generally absent. There was evidence of a severe degeneration of the villous epithelium, with shortening of cells, loss of nuclear polarity, abnormal staining of cytoplasm, indistinct cell borders, reduction or loss of the PAS-positive striated border, and, particularly at the tips, necrosis. With one exception, the cellular exudate in the tunica propria consisted almost exclusively of large macrophages and only occasional segmented leukocytes. Virtually no eosinophils, plasma cells or lymphocytes were seen. In the only animal sacrificed while outwardly still healthy there were many neutrophils present. Large amounts of Feulgen-positive pyknotic nuclear debris, amorphous and violaceous precipitate, and evidence of inflammatory edema were noted in the lamina propria, obscuring the reticular framework. There was distention of lymphatics and a marked venous hyperemia. Micro-organisms were noted in the lamina propria, particularly in areas of ulceration. The stomach and colon were free of ulceration and cellular inflammatory exudate. There was, however, evidence of epithelial degeneration and hypersecretion of mucus (Fig. 8).

In describing lymphatic tissue, we have utilized the expression "solid secondary follicle" to indicate a fairly small nodule composed of lymphocytes; by its compactness this is distinctly separated from the surrounding lymphoid tissue. We have distinguished 3 types of "secondary follicles with reactive centers" and have designated them "early," "highly active," and "terminal," using the criteria of Conway[5] and Ringertz and Adamson.[6] Intermediate forms have been frequent.

When compared with lymph nodes in CONV animals, the over-all

size of nodes in the NGF group was distinctly small, the cortical sinuses were collapsed and the cortex was not sharply delineated from the medulla. The cellularity of the cortex and medullary cords was reduced and approximated that in neonatal animals. In NGF animals, lymphocytes were distributed diffusely in a sheet-like manner or in ill-defined, larger aggregates of more compactly arranged cells occasionally designated "primary follicles." Secondary follicles, when present, were usually of the "solid type" (Fig. 9). Occasionally, early reactive centers surrounded by narrow rims of compactly arranged lymphocytes were seen. Concentric lamination of lymphocytes was rarely encountered.

In the NGF group, reticulum cells were readily discernible in the narrow medullary cords because of the relative reduction of lymphocytes. Littoral cells were rather inconspicuous. The medullary sinuses contained a varying but never more than a moderate number of lymphocytes and monocytes. Macrophages, neutrophils and eosinophils were rare, and mature plasma cells were not seen. Red cells, free and phagocytized, a constant feature in CONV guinea pig lymph nodes, were also observed in the germ-free groups. Oral bacterial challenge had no apparent effect on the numbers of red cells in sinusoids or the degree of erythrophagocytosis. Mitotic figures were rare and were not confined to follicles. There were occasional lymphoid aggregates and solitary follicles in the intestine of NGF animals. These were much smaller than in the CONV group and rarely exhibited reactive centers, frequent in CONV animals.

The lymph nodes in the GF-C group were generally slightly larger than in the NGF animals; the cortex was slightly thickened, and the medulla more cellular. Follicles were prominent and increased in number though not greatly enlarged in size. The majority of the follicles were of either the solid secondary or the early reactive type.

In contrast to the NGF group, in which small lymphocytes outnumbered other types by far, medium-sized and large lymphocytes were prominent in the GF-C animals. Here, too, large mononuclear cells which had phagocytized cellular debris appeared singly within follicles, in the interfollicular pulp and in the medullary cords. Cortical reticulum cells and medullary cords were enlarged and, at times, formed a sheetlike network pierced by narrowed sinusoids. Littoral cells were enlarged. An occasional mature plasma cell with abundant cytoplasm was seen; eosinophils were rare. In the sinusoids, monocytes constituted the most common cell type. Lymphoid aggregates had the same number and phase of cycle of both GF-C and CONV guinea pigs. The secondary follicles in GF-C animals were, however, smaller, and highly active centers were not evident.

Lymph nodes in GF-C&S animals were larger than those in NGF animals. The follicles were predominantly of the early and highly active type, showing many mitotic figures and active cytophagocytosis. Solid secondary follicles were rare, and only a few follicles with reactive centers approaching the terminal phase were noted. Despite the high degree of lymphopoietic activity in follicles, the number of cells in the cortex and medulla appeared reduced when compared with nodes in the GF-C group, and lymphocytes were distributed in a loosely constructed reticular framework. The appearance suggested a partial emptying of nodal lymphocyte population. While all types of lymphocytes were involved, the small lymphocyte was particularly affected. In the GF-C&S group, reticulum cells had greater reactive pleomorphism than in the GF-C group. They appeared in sheets, separating follicles from each other. The medullary cords were thickened and populated predominantly by mononuclear cells and lymphocytes. Mature plasma cells were usually present in small numbers; eosinophils were rare. Cortical and medullary sinusoids were moderately distended by lymph containing a moderate suspension of macrophages and lymphocytes. The intestinal lymphoid aggregates were slightly larger than in GF-C animals and contained secondary follicles with highly active centers.

Mesenteric lymph nodes in the GF-SI group (Figs. 10 and 11) differed from those in all other groups, having marked hyperemia and reticulum cell hyperplasia. The cortical and medullary sinuses were severely distended and, in addition to neutrophils, contained a predominance of macrophages which were markedly swollen and laden with cellular debris. Lymphocytes were, in general, medium-sized and were more loosely arranged, as though washed out of the reticular net. Eosinophils and plasma cells were not encountered. Follicles were small and without reactive centers. In contrast, lymphoid aggregates in the ileum and cecum contained large secondary follicles with highly active centers (Fig. 12). Cytophagocytosis of nuclear debris was marked, particularly in the reactive centers. In mediastinal nodes of GF-SI animals the reaction was similar in quality to that described in the mesenteric nodes, but was of distinctly less intensity.

The adrenals in GF-C and GF-C&S animals differed from those of the NGF group in the following manner: The zona glomerulosa was not as sharply defined; cells in this zone were larger, and their cytoplasm was slightly vacuolated. Mitotic figures were more numerous, especially in GF-C animals where there were as many as 5 to 6 per high-power field; there was also an increase of cellular pleomorphism in the zona fasciculata, variation in the amount of cytoplasmic lipid, and necrosis of cortical cells. Adrenals in many of the GF-SI animals showed marked

venous hyperemia, focal hemorrhagic necrosis, and reduction of lipid content; there was no increase of mitotic figures over those noted in the NGF group.

Discussion

The conventionally reared guinea pig is resistant to oral challenge with *S. flexneri*. Only when the resistance of the animal is modified by starvation or injection of carbon tetrachloride does infection supervene.[7] In this regard, the animal resembles the human subject with bacillary dysentery, in whom a "dysentery milieu"[8] induced by such factors as cold, malnutrition, physical stress and nonspecific gastroenteritis has been postulated.

On the other hand, the germ-free guinea pig succumbs rapidly to Shigella infection. The course of the disease and the extent of intestinal involvement are similar in GF-SI and in a group of modified CONV animals investigated by us.[9] In these, extensive small intestine involvement occurred; this resembled the acutely fatal form of the disorder especially observed in children, in whom involvement of the entire gastrointestinal tract has occasionally been noted.[10] In general, tissue alteration is limited to the large intestine in man and in those modified CONV guinea pigs which do not succumb to the acute ailment.

Letterer[11] found that Shigella endotoxin had no effect when introduced into the intestinal lumen or dropped directly onto the intact mucosa in conventionally reared mice. The intestinal epithelium in the conventionally reared adult mouse is impervious to macromolecules of the size of endotoxin;[12] the absorptive capacity of the intestinal lining in germ-free guinea pigs is as yet unknown. Letterer found that the same endotoxin administered intravascularly led to vascular insufficiency, alteration of the mucosal pattern and ultimately to ulceration. We observed similar changes. It would seem that after the disease process had been triggered, the tissue changes ran a similar course in endotoxin-treated mice and in orally infected, modified conventional and also germ-free guinea pigs.

Prior introduction of *E. coli* via the oral route prevented the occurrence of mucosal lesions in the germ-free guinea pig exposed to dysentery bacilli. Shigella organisms were generally absent from the intestinal content and tissues of GF-C&S animals at the time of necropsy.[4] Colicins apparently do not play a role in the disappearance of Shigella organisms since the strain of *E. coli* we have used does not produce this type of antibiotic agent. *E. coli* is not constantly present in the intestinal flora of the CONV guinea pig,[13] and yet this animal is resistant to Shigella. Possibly other enteric organisms may exert an antagonistic effect on

S. flexneri. On the other hand, LaBrec and Formal,[14] employing fluorescent antibody techniques in modified CONV guinea pigs, demonstrated very early invasion of *S. flexneri* in the lamina propria of the duodenum and jejunum, a region of the intestine usually free of *E. coli*. This suggests that the protective effect of *E. coli* cannot be explained entirely by bacterial antagonism.

Moog and Thomas[15] and Halliday[16] demonstrated the effect of adrenal cortical steroids on the maturation of the intestinal epithelium. The introduction of *E. coli* into the intestinal tract of the NGF guinea pigs resulted in modification of the mucosa. The alterations in the GF-C animal were not confined to the gut but included lymphoid tissues and the adrenal cortex. These alterations are taken as evidence of a functional adaptation which may reflect a physiologic and immunologic change in the defense mechanism contributing to the resistance of the host to a subsequent Shigella infection. The existence of immunity strictly localized to the intestinal mucosa without a systemic component has not been proved.[17] In our experimental model, resistance to Shigella infection was associated with the presence of viable *E. coli* organisms. As we have pointed out,[4] protection against the almost universally fatal Shigella infection has not been afforded by prior feeding of killed *E. coli* or killed *S. flexneri* or of live lactobacilli, organisms normally present in the intestine of CONV guinea pigs.[18] Only prior parenteral injection of killed *S. flexneri* has on rare occasions been associated with protection against a subsequent Shigella infection.

The starting point of germ-free research was the investigation of the concept that the intestinal flora was needed for the preservation of life. However, once the rearing of germ-free animals was readily achieved, more attention was paid to the lymphatic tissues to the exclusion of determining the morphologic details in the intestinal mucosa of such animals. We have demonstrated that, depending on the intensity and duration of an orally introduced stimulus, a distinct type of alteration in the intestinal mucosa may be observed. For example, there is a characteristic pattern in the intestinal mucosa of each of the following groups: NGF, GF-C, GF-C&S, CONV and GF-SI animals. There is an anatomic progression of alteration in the various components of the mucosa as one proceeds from the NGF to the GF-SI group (Table I). The intestinal tract in the NGF animal has received the smallest number of stimuli and has the simplest mucosal pattern. The stimuli consist of dietary components including dead bacteria in the sterilized food, possibly other antigenic substances in the tank environment, and mechanical irritation resulting from the passage of food. The controlled addition of live bacteria produced, in stepwise manner, greater histologic changes in GF-C,

GF-C&S and GF-SI; the CONV group, receiving all the stimuli of natural life, exhibited more alteration than seen in the GF-C&S group. Stimuli of nonphysiologic nature produced severe alterations of the mucosal architecture, as noted in the GF-SI group.

Similar features have been observed in other experimental enteric infections, and the small bowel mucosa may assume an appearance not unlike that seen in some instances of human sprue.[9] The modifying influence exerted by the intestinal flora affected both the small and large

TABLE I

MUCOSAL PATTERNS IN SMALL INTESTINE

	NGF	GF-C	GF-C&S	CONV	GF-SI
Thickening of villi	o	+	+	++	++++
Shortening of villi	o	o	o	+	++++
Lengthening of crypt glands	o	o	+	++	++++
Reduction of mucus content in crypts	o	+/++	+	+++	++++
Degenerative changes in epithelial lining	o	+	+	+	++++
Cellularity of lamina propria	o	+	+/++	+	++++

NGF = germ-free guinea pig, unchallenged by bacteria.
GF-C = germ-free guinea pig, challenged with *E. coli*.
GF-C&S = germ-free guinea pig, challenged with *E. coli* and Shigella.
CONV = conventionally raised adult guinea pig.
GF-SI = germ-free guinea pig, Shigella infected.

bowel. However, in the small intestine, the correlation between the degree of epithelial degeneration, lamina propria inflammation, and the ratio of villous to nonvillous portions of the mucosa and the depth of the crypt glands was more evident than in the cecum, where villi are not well developed and become obliterated in CONV animals.

The histologic pattern of the normal intestinal mucosa in the CONV animal represents only a segment of the spectrum of possible responses and reflects the results of past and present stimuli (Table I). A synergism exists between stimulus and responding tissue which produces what is considered to be the normal adult histologic pattern in the intestine. The mucosa is very labile in its reaction to injury, and in its response all component parts participate as one unit; rapid and profound structural alterations may occur.

Using newborn guinea pigs, Gyllensten[18] established the role of bacteria in the postnatal development of lymphoid tissue. Our observations in the germ-free animal indicate that bacteria also play a major role in the development of the definitive pattern of the intestinal mucosa. In this regard, they act as postnatal morphogenetic stimuli. This concept may well apply to other animal species, including man. The bacterial flora is not the exclusive morphogenetic stimulus and, like the lymphatic tissue,

intestinal development will be influenced by dietary and hormonal factors.

Whether germ-free animals can respond to antigenic stimuli and whether the absence of reactive follicles is due to the malnourished state of such animals have been questioned.[19] We and others[20–22] have observed reactive centers in antigenically challenged germ-free animals. Miyakawa, using the Gifu uniform strain of guinea pigs, found no reactive centers in his NGF animals, confirming Glimstedt's original observations.[23] However, we concur with Thorbecke that the germ-free animal is only quantitatively but not qualitatively different from CONV animals in its degree of exposure and response to various antigenic stimuli, and that occasional reactive centers are seen in the lymph follicles of NGF animals. Species and strain differences of the animals employed, seasonal variations and minor modifications of the diet used in raising germ-free animals may affect the response of lymphatic tissue. Other factors which may influence our observations are the time interval following introduction of the bacteria and the age at which the animals are examined.

As pointed out by Miyakawa, there are differences in the response of lymphoreticular tissue in conventional and germ-free animals. These differences manifest themselves in the plasma cell response and in the development of secondary follicles and their reactive centers. Both of these are inconspicuous and their appearance delayed in GF animals. Our animals showed significant variations in the time interval between the several bacterial challenges and the appearance of secondary follicles and in the size of the reactive centers produced. Both features seemed to correlate with the intensity of stimulation. Secondary follicles with reactive centers appeared sooner and were larger following Shigella infection. GF-C&S animals which were exposed longest to bacteria showed the highest degree of lymphoid tissue maturation.

Summary

1. Germ-free guinea pigs developed an acute ulcerative enterotyphlitis following oral challenge by *S. flexneri;* this was fatal within 48 hours. In contrast, conventionally reared guinea pigs were not susceptible to this infection.

2. Germ-free guinea pigs were protected against fatal Shigella infection by the prior oral introduction of *E. coli.*

3. The intestinal mucosa of the germ-free guinea pig resembled that of the prenatal pig but differed from that of the conventionally reared animal by showing near absence of inflammatory cells in the lamina propria, distinctly shallow crypt glands lined by a high proportion of

markedly distended goblet cells, absence of degenerative changes in the epithelium lining the villi, taller and more delicately shaped villi in the small intestine, and a villous pattern in the cecum.

4. Bacteria appear to exert a morphogenetic stimulus. Following oral introduction of *E. coli*, the architecture and histologic pattern of the bowel, within a matter of a few weeks, approached those seen in conventionally raised animals.

5. A more intense stimulation by Shigella infection produced an accentuation of these features, with total loss of goblet cells, marked degenerative alterations of mucosal epithelium, marked deepening of crypt glands, shortening and blunting of villi, and, occasionally, obliteration of the villous architecture. Highly active secondary follicles in the lymphoid aggregates of the intestine were seen only in this group.

6. The response of lymphoid tissue to oral bacterial challenge was influenced by the germ-free state; graded intensity of response related to the proximity of the irritant. The phase of the reactive centers in secondary follicles and the number of reticulum cells and immature lymphocytes depended primarily upon intensity of stimulation and less on the duration of exposure. The duration of a stimulus affected the over-all maturation of lymphoid tissue.

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[*Illustrations follow*]

Legends for Figures

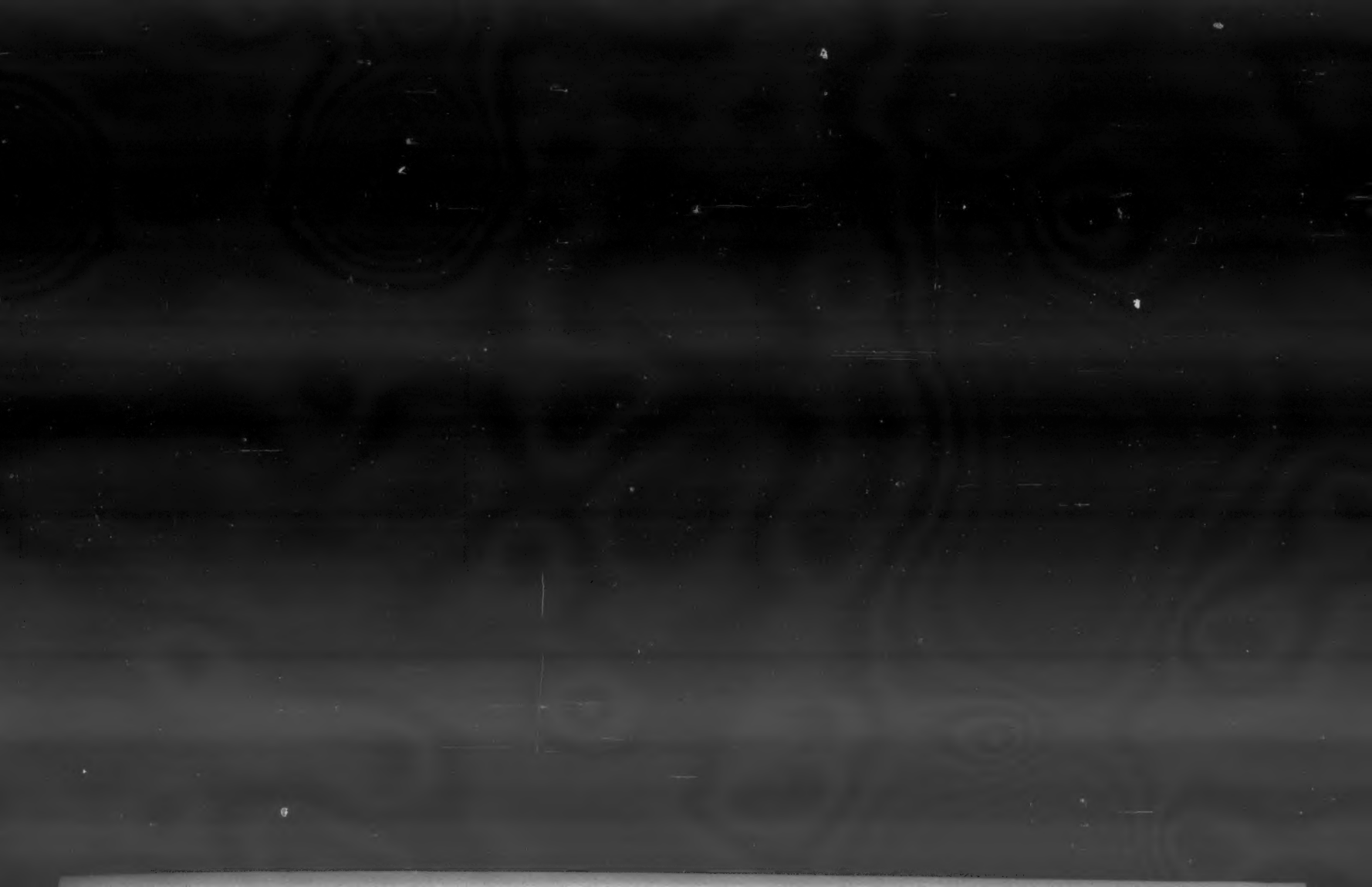
Illustrations were prepared from sections stained with hematoxylin and eosin. Abbreviations utilized: NGF = germ-free guinea pig, unchallenged by bacteria; GF-C = germ-free guinea pig, challenged with *E. coli;* GF-C&S = germ-free guinea pig, challenged with *E. coli* and Shigella; CONV = conventionally reared adult guinea pig; GF-SI = germ-free guinea pig, Shigella infected.

Fig. 1. Ileum, 42-day-old CONV guinea pig. Note the ratio of villous to the nonvillous portion of the mucosa, the depth of crypt glands, the shape of villi, the distribution of goblet cells and the cellularity of the lamina propria. × 158.

Fig. 2. Ileum, 42-day-old NGF guinea pig. Note the altered ratio of the villous to the nonvillous portion of the mucosa, not affecting the height of villi. There are a predominance of markedly distended goblet cells in the shallow crypt glands and a sparsity of cells in the lamina propria. × 178.

Fig. 3. Ileum, 48-day-old GF-C guinea pig. Note the expansion of the nonvillous portion of the mucosa; the crypt glands have deepened, and there is a reduction of goblet cells. Increased cellularity in the lamina propria and focal degeneration of the epithelium at the villous tips are evident. The mucosa now approaches the pattern found in CONV animals (Fig. 1). × 158.

Fig. 4. Ileum, 43-day-old GF-SI guinea pig. Villi are blunted and thickened. There are flattening of the surface and elongation of crypt glands, producing a sprue-like pattern. Epithelial degeneration is associated with micro-ulceration. Note the cellularity of the tunica propria and the absence of goblet cells. × 156.



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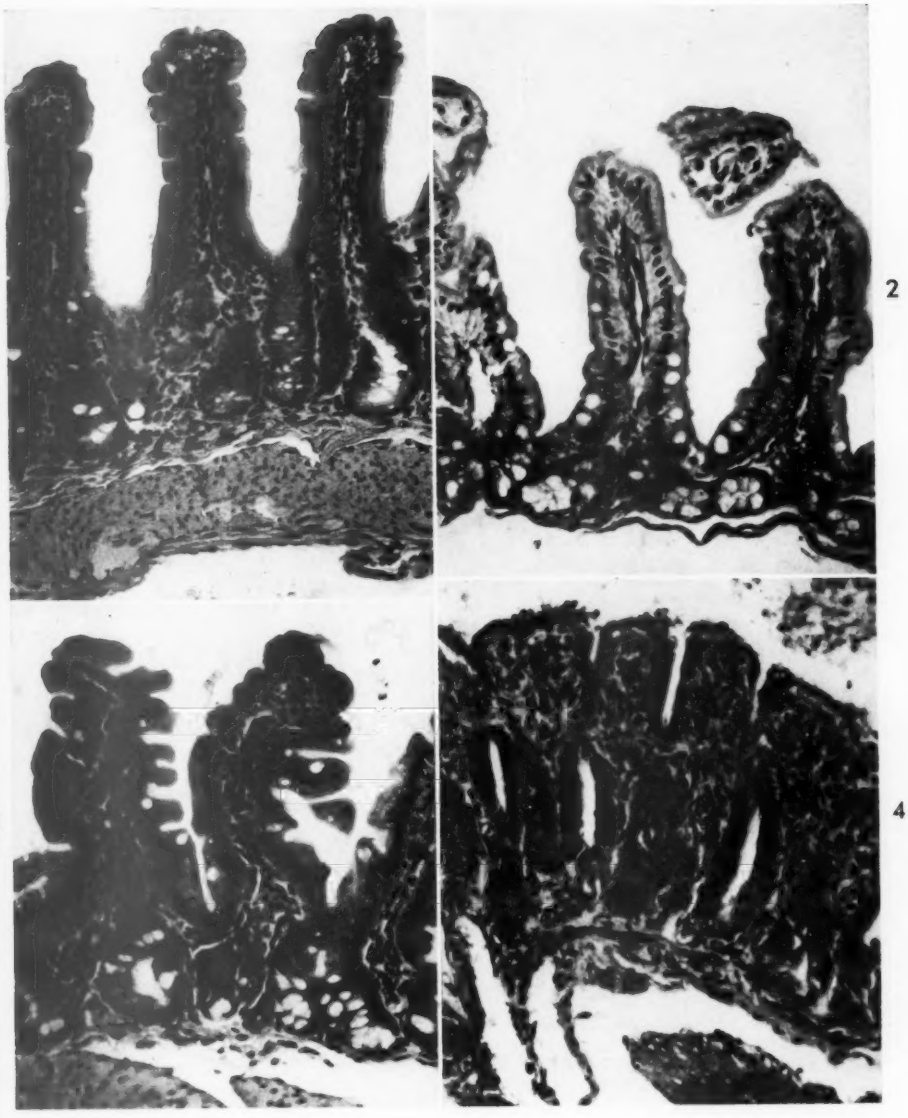

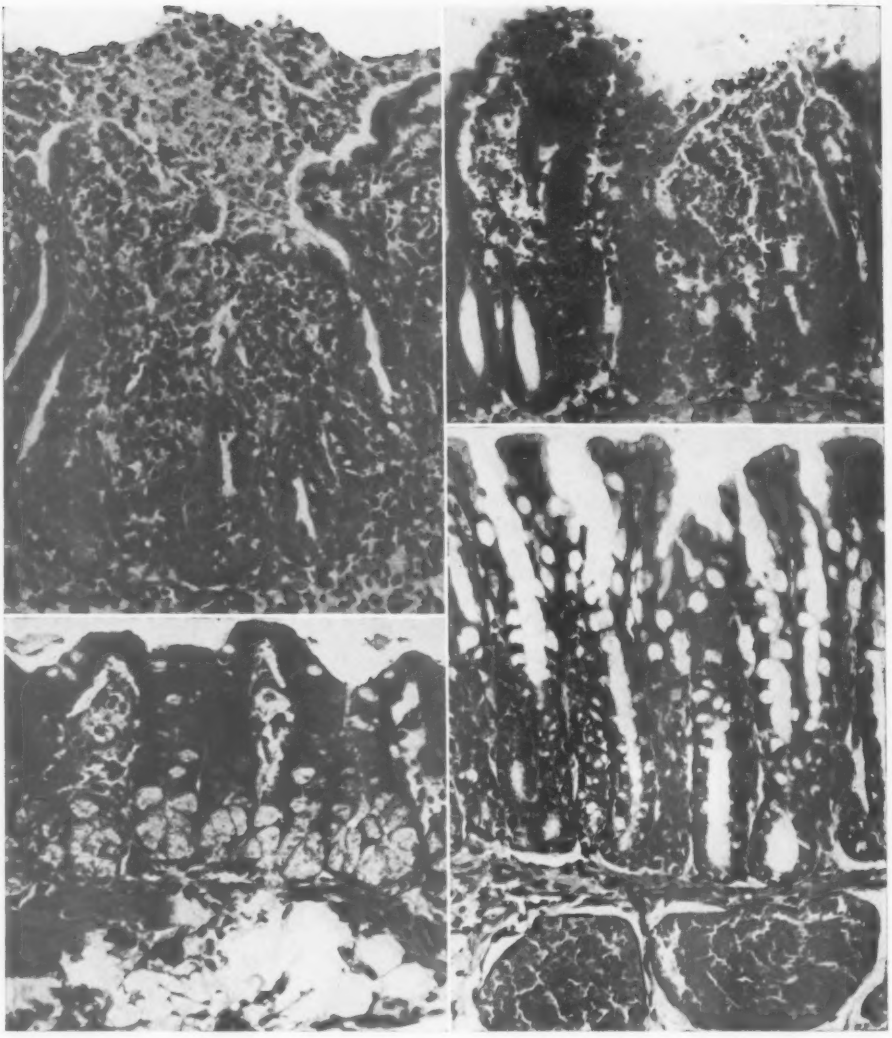

FIG. 5. Ileum, 43-day-old GF-SI guinea pig. Acute ulcerative enteritis with pseudo-membrane formation is manifest. Goblet cells are absent and there is marked deepening of crypt glands. × 158.

FIG. 6. Cecum, 43-day-old GF-SI guinea pig. Extensive necrosis is accompanied by marked epithelial degeneration, inflammatory cell infiltration, deepening of crypt glands and absence of goblet cells. × 158.

FIG. 7. Cecum, 42-day-old NGF guinea pig. Goblet cells are markedly distended. × 158.

FIG. 8. Colon, 43-day-old GF-SI guinea pig. Goblet cells are decreased, especially in basal portion of crypts. Crypts are distended with mucus. There are epithelial degeneration, severe venous engorgement in the submucosa, and absence of an inflammatory cellular response. × 158.

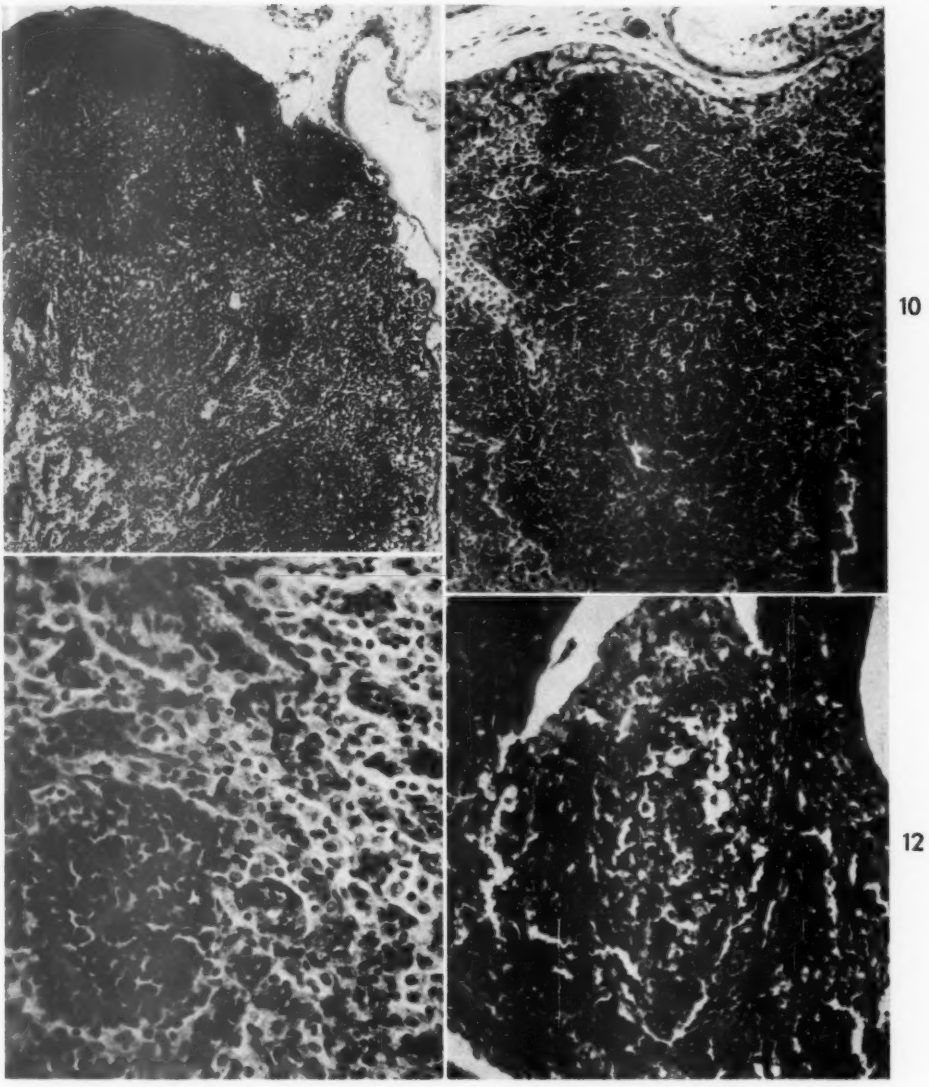

FIG. 9. Mesenteric lymph node, 42-day-old NGF guinea pig. Secondary follicles are solid; the cortex blends with the medulla. Peripheral and intermediary sinuses are collapsed. × 52.

FIG. 10. Mesenteric lymph node, 43-day-old GF-SI guinea pig. There are marked dilatation of afferent lymphatics, severe hyperemia, and relatively small follicles without reactive centers. Sinuses are distended with mononuclear cells. × 80.

FIG. 11. Mesenteric lymph node, 43-day-old GF-SI guinea pig. Hyperemia of the medulla is apparent; medullary cords are largely devoid of lymphocytes; sinuses are distended and filled with mononuclear cells. × 179.

FIG. 12. Solitary follicle, ileum, 43-day-old GF-SI guinea pig. A large, highly active center has a mantle zone best developed at the base of the follicle. × 145.

SPLENIC WHITE PULP ALTERATION AFTER ANTIGEN INJECTION: RELATION TO TIME OF SERUM ANTIBODY PRODUCTION

C. C. Congdon, M.D., and T. Makinodan, Ph.D.

From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.*

In spite of rather clear indications from the older literature that the germinal center cells of the organized lymphatic tissues are of importance in understanding the immune mechanism, relatively little attention has been paid to them in recent years. Österlind [1] was one of the first histologists who specifically studied changes in the germinal, or reaction, centers of these tissues during antibody formation in experimental animals. He summarized his findings as follows: "These observations corroborate in all ways the view that reaction centers function as immunizing organs, and from a general biological point of view it does not seem overbold to set up the theory that they are specially designed antibody producers." Österlind's work was an extension of the much earlier investigations by Hellman and White [2] on the immune function of the reaction centers in lymphatic tissue. That marked changes occurred in germinal center tissue during an immunization process was known as early as 1918.[3] Ringertz and Adamson [4] have reviewed much of the literature on this subject.

Micro-organisms or their metabolic products were being studied in most of these early experiments, and many workers apparently felt that the cellular changes were a response to toxic substances in the injected material.[5] Recent investigations, however, required that the theory of the toxic origin of the cellular alterations be reconsidered. Amano,[6] however, seemed to believe that the germinal center changes were indicative of an irritative phase occurring immediately after antigen injection, separable from the antibody-producing stage of cellular change.

Newer studies have included the evidence presented by Ortega and Mellors [7] that germinal center cells contain gamma globulin. In addition, White [8] has demonstrated specific fluorescent-labeled antibody in the germinal centers of rabbit lymph nodes after secondary response.

We have been independently investigating the bizarre cellular changes that occur in lymphatic tissues of lethally irradiated mice given foreign bone marrow cells or spleen cells intravenously [9,10] and observed that

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an equally remarkable series of cellular alterations took place when the recipients had not been exposed to X rays. The changes seemed to be best explained as germinal center changes.[11–14] Similar changes in lymphatic tissues of normal mice given a wide variety of antigenic materials, including nontoxic crystalline protein antigens, were also observed.[13–14]

The fundamental alteration was a proliferation of germinal center cells and their apparent transformation into antibody-forming cells. When this growth took place within the organized center, it gave the appearance of a hyperplastic center, as described by Österlind[1] and others. When it took place in a dissociated manner within the white pulp (in the case of the spleen) or lymph node cortex, the intact germinal centers could not be found. Cellular changes in the spleen red pulp and lymph node medulla during an immune process presumably result from the migration and proliferation of the derivatives of germinal center cells. We suppose that these germinal center changes represent the inductive phase of antibody formation since they develop within a few hours after antigen administration.

In the present work, groups of mice were given sheep red cells intravenously and then killed at daily intervals to obtain serum for measuring sheep red cell agglutinins. The spleens were fixed for histologic study so that changes in the germinal centers could be compared to the time of production of titratable serum antibody.

Material and Methods

Fifty female BC_3F_1/Cum ($C_{57}BL$/Cum ♀ × C_3H/Anf Cum ♂) mice about 12 weeks of age were used in the first experiment. They were allowed free access to food and water and were caged in groups of 5 to 10 animals. Five of the 50 animals served as normal controls for body weight, spleen weight, and spleen histology. The remaining 45 mice were given an intravenous injection of 1 ml. of 10 per cent washed sheep red blood cells. Twenty-five of these were then killed in groups of 5 each for 5 days after the injection. These 25 mice were the primary injection group. Blood serum was collected for determination of antibody against sheep red cells. Individual body weights and spleen weights were taken at killing, and the spleen was fixed in a Zenker-formol solution for later histologic study of sections stained with hematoxylin and eosin. An additional section from one spleen in each group killed daily was stained with methyl green pyronine. Three of the remaining 20 injected mice were killed 6 weeks after the primary injection, and served as controls for the secondary injection group which consisted of 17 mice that received an additional intravenous injection of 1 ml. of 10 per cent sheep red cells. Three to 4 of this group of mice were then killed each day for 5 days after the second administration of sheep red cells. Measurements were made and materials collected as in the primary injection group.

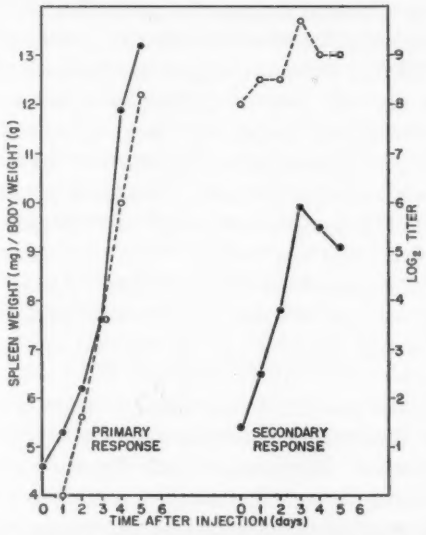
In the second experiment, 75 mice were given 1 ml. of 1 per cent sheep red cells intravenously. Each day thereafter for 15 days, 5 mice were killed for serum antibody determination, and the spleens were fixed for histologic study as in the first experiment.

The method of determining antibodies (expressed as $\log_2$ titer) against sheep red cells in mouse serum was that previously described by Makinodan, Gengozian and Congdon.[15]

Spleen weight changes were expressed as the ratio of spleen weight in mg. to body weight in gm.

Results

The spleen-body weight ratio and the serum antibody production of the primary and secondary injection groups given 1 ml. of 10 per cent sheep red cells are shown in Text-figure 1. Although the spleen weight returned to near-normal values at 6 weeks after the primary injection, the



Text-figure 1. Spleen-body weight ratio and serum antibody production after primary and secondary injection of 1 ml. of 10 per cent sheep red cells.
•———•, spleen weight ratio; o – – – o, titer.

serum antibody production was still at very high levels and relatively little increase occurred with the second injection of sheep red cells.

Hematoxylin and eosin stained sections from the spleens of the 5 normal mice all showed the presence of germinal centers. Under low magnification a total of 130 germinal centers of variable size could be distinguished in the antero-posterior longitudinal sections through the spleen white pulp. The number seen for each individual spleen section varied from 21 to 32. Figures 1 and 2 show an example of one of the larger centers. The methyl green pyronine stain on one spleen showed marked pyroninophilia in the germinal center cells with greater pyronine staining in the inner portion than in the outer portion of the center nearest the red pulp. Pyronine staining was observed in some of the blood-forming cells in the red pulp. In 3 of the mice a few clumps of young plasma cells or antibody-forming cells were seen at the white pulp

margin. These were thought to represent a part of the background immune response to unknown antigenic stimuli present in most so-called normal animals in one portion or other of the lymphatic system. This amount of "noise" in our material, like the variation in size of the germinal centers, does not seem to be a serious matter in making the histologic analysis of changes occurring during the immunization process.

Primary Injection Group

None of the 15 spleens examined during the first 3 days (5 per day) after the primary injection of sheep red cells possessed organized germinal centers. Marked cellular changes were present in the 5 spleens examined on the first day, 24 hours after the injection. Besides loss of organized germinal centers in the white pulp, there was marked reduction in the number of tissue lymphocytes, and the area around the central artery of the white pulp had an edema-like quality, with separation of cellular elements (Fig. 3). Increased numbers of tingible bodies containing much cell debris could be identified. Newly formed cells were present throughout the white pulp from the central artery to the marginal zone. These cells had a large vesicular nucleus with prominent acidophilic nucleolar material and basophilic cytoplasm that was heavily pyronine-staining. Throughout the text we designate these cells as "Ab-forming cells," following the practice initiated by Wissler, Fitch, la Via and Gunderson [16] for denoting quite similar cells observed in the rat spleen after antigen injection. Numerous mitotic figures were present in the white pulp; the cytoplasm of the cells was stained with pyronine. The red pulp at this interval was congested and contained many granulocytes. There was a reduced number of blood-forming cells. The marginal zone cells were swollen. Some of the granulocytes infiltrated the white pulp and appeared to make up part of the cell debris in the tingible bodies.

Blood serum from the animals whose spleens had such marked changes contained no antibody to sheep red cells at the 24-hour interval (Text-fig. 1).

Spleens taken 2 days after the primary injection had many focal collections of pyronine-staining Ab-forming cells at the marginal zone and extending out into the red pulp, often along the trabeculae (Figs. 4 and 5). At this interval, serum antibody against sheep red cells was present. Congestion of the red pulp was greatly diminished, as was granulocyte infiltration, and tingible bodies were difficult to find. Active blood cell formation was now present in the red pulp.

At day 3 the proliferation of Ab-forming cells in the red pulp was very marked, and some of them resembled plasma cells. The serum titer against sheep red cells was greater than that of the previous day. Through

the third day the changes suggested that the antigenic material had somehow activated the pyronine-staining germinal center cells so that a mass of dividing cells of the Ab-forming type moved out from the white pulp into the red pulp where they continued to increase in numbers and also probably continued their transformation into plasma cells. The appearance of some sections suggested another mechanism for getting Ab-forming cells into the red pulp. In this case the white pulp appeared to open up into the red pulp, and the distinction between white and red pulp was lost (Fig. 6). From the time of the appearance of the cells in the red pulp there was an increasing amount of serum antibody.

At days 4 and 5 restitution was apparent in the germinal centers of all the spleens examined. Nearly every section through the white pulp showed hyperplastic centers with prominent tingible bodies (Fig. 7). Large numbers of pyronine-staining, Ab-forming cells were present in the red pulp. The cytoplasm of these cells appeared more eosinophilic than at earlier intervals. Some plasma cells were seen, and there were many mitotic figures.

Significant lymphocyte re-accumulation had not yet taken place in the white pulp when this experiment was terminated.

Secondary Injection Group

Six weeks after the primary injection, the spleens of the mice used as controls for the secondary injection had normal germinal centers and white pulp. Twenty-three to 29 centers could be counted under low magnification in a single spleen section. The serum of these 3 mice gave a very high titer to sheep red cells although the spleen weight was near normal. There were some plasma cell collections in the red pulp; these were somewhat more numerous than were seen in the spleens of the normal, uninjected mice.

During the first 3 days after the secondary injection, the histologic appearance of the spleen was characterized by essentially the same series of changes as were present after the primary injection, with the exception that some intact germinal centers were present. Thirty-nine centers were counted in the spleens from 12 mice, with the number varying from none to 12 in a section of spleen.

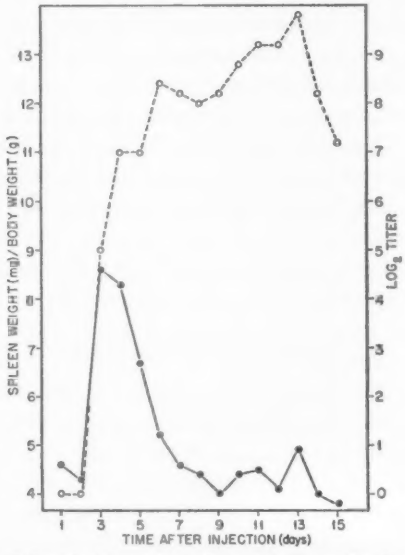
In the day 4 and 5 sections, there was a great amount of Ab-forming cell proliferation in the red pulp and some in the white pulp, but a uniform restitution of germinal centers in the white pulp, as seen after primary injection, was not observed. In some spleens, hyperplastic germinal centers did appear, but in others the white pulp contained dissociated masses of Ab-forming cells.

The spleen weight changes after the third day were also quite variable

from animal to animal, giving a much lower average spleen weight than seen on days 4 and 5 after the primary injection.

In the second experiment, with the much smaller antigen dose, expected changes in spleen weight and the appearance of serum antibody occurred (Text-fig. 2).

Histologic examination of the spleens in these mice revealed the same sequence of cellular changes as found with the larger antigen dose in the primary response. Quantitatively, however, the response was much re-



TEXT-FIGURE 2. Spleen-body weight ratio and serum antibody production after primary intravenous injection of 1 ml. of 1 per cent sheep red cells.
•———•, spleen weight ratio; o – – – o, titer.

duced compared to that with the larger amount of antigen. Not all germinal centers disappeared. The pyronine-staining cells of the plasma cell series were present in the red pulp on day 3 when antibody was first detected in the serum, but the number of these cells in the red pulp was much less than with the larger amount of antigen. Hyperplastic germinal centers with many tingible bodies were present in the day 4 spleens, and somewhat enlarged germinal centers were present throughout the 15-day period of observation. The Ab-forming cells in the red pulp had nearly all gone in the spleens examined on day 7 and thereafter.

DISCUSSION

These studies show very clearly that after sheep red cells are injected intravenously in the mouse, extensive cellular changes take place in the

white pulp of the spleen before cellular proliferation can be detected in the red pulp and before serum antibody develops. The amount of cellular alteration in the white pulp, as well as that in the red pulp, was greater with the larger of the 2 antigen doses studied.

The character of the white pulp change was complex, but the most striking observation after primary antigen injection was loss of germinal center cells accompanied by extensive proliferation of Ab-forming cells throughout the white pulp. This process lasted about 3 days, followed by a hyperplastic regeneration of germinal centers.

Changes in tingible bodies, tissue lymphocytes, stroma, and marginal zone cells of the spleen white pulp were also noted, but their significance for the immune reaction is not known.

The change in the spleen that seemed to correlate best with the appearance of serum antibody was the development of Ab-forming cells of the plasmacytic series at the margin of the white pulp and in the red pulp. This correlation of histologic structure with serum antibody is well known from other work.[16] However, in our second experiment, plasma cells had largely disappeared from the red pulp in 7 days, but high antibody titers were found in the serum throughout the 15-day period of observation. The possible sources of the continued antibody production could be from Ab-forming cells disseminated throughout the lymphatic tissues of the animals, or, more likely, from the hyperplastic germinal centers of the spleen white pulp. To prove this latter contention would require different techniques from those used here, but the demonstration by White[8] of fluorescent-labeled specific antibody in germinal center cells during a secondary response might be cited in support of the idea.

Perhaps the most important issue to be discussed is whether the large, newly formed cells in the white pulp actually give rise to the Ab-forming cells of the plasmacytic series that are later seen in great numbers in the red pulp. The sequence of histologic events seems to suggest that this is the case, but even more suggestive is the fact that the germinal center cells, the newly formed cells in the white pulp, and the Ab-forming cells in the red pulp are all pyronine-staining. This natural marker appears to be only associated with the cells just mentioned in the part of the spleen showing cell proliferation after antigen injection.

Amano[6] had a different point of view about the origin of the proliferating cells in the white pulp and red pulp after antigen injection. Even though they were both pyroninophilic, he felt they had a separate origin and that their electron micrographs differed. Amano believed plasma cells came from blood vessel adventitial cells. Perhaps further studies will be needed to finally prove whether or not Ab-forming cells migrate from the white pulp to the red pulp. One interesting observation in the

present work was the finding that the white pulp disappeared, and, in effect, became red pulp filled with Ab-forming cells (Fig. 6). Such observations suggested that migration from white pulp to red pulp occurred at special regions of white pulp.

The secondary activity evoked with 1 ml. of 10 per cent sheep red cells indicated that some germinal centers were not responding. Others went through the same sequence of changes that were seen after the primary antigen injection. We interpret the unresponsiveness to mean that germinal centers were already making antibody to sheep red cells or possibly some other unknown antigen, but the point needs further study. From observations of the kind seen in the present experiments in which proliferation of dissociated germinal center cells occurred, followed by hyperplastic regeneration of germinal centers, one can conclude that two types of proliferation of germinal center cells are possible; one in the intact germinal center and one without an intact germinal center. The experimental variables controlling the mode of growth of germinal center cells still need to be determined.

The ideas that changes in the white pulp represent either toxic damage to cells, as Wissler, Fitch and la Via [5] suggested, or an irritative phenomenon, as Amano [6] thought, seem untenable in view of the uniform finding of these changes when antigens are injected into animals of different species. It seems most likely that the very early changes in spleen white pulp are the inductive phase of antibody formation. Ontogenetic evidence also indicates that the spleen white pulp and the lymph node cortex are more important than the red pulp and the medulla as sites of the initial response of the immune mechanism. In the mouse, for example, spleen red pulp is present during fetal life, but an immune function does not appear until after birth when the white pulp develops. Somewhat similarly, radiation profoundly damages the immune response, and the major cellular change in spleen and lymph nodes is in the nodular portions of these organs rather than the medullary and red pulp elements.

Summary

Marked cellular alterations in the spleen white pulp occurred in mice given sheep red cells before serum antibody production could be detected. These changes also preceded the appearance of cellular proliferation in the red pulp.

The histologic evidence favored the idea that the Ab-forming cells of the plasmacytic series in the red pulp were derived from the proliferating cells in the white pulp, and these in turn seemed to be derived from dissociated germinal center cells.

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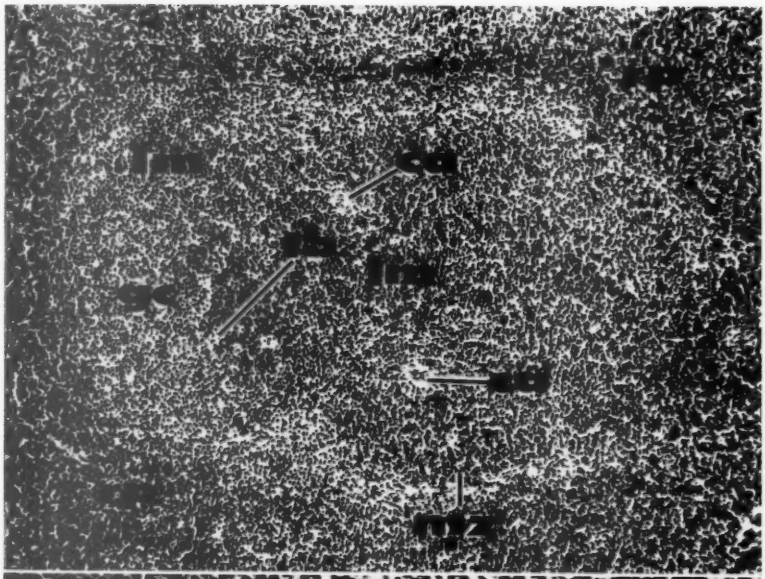
[*Illustrations follow*]

Legends for Figures

Photomicrographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. White pulp of the spleen in a normal mouse showing a germinal center (gc) containing several tingible bodies (tb). The germinal center is surrounded by a mass of lymphocytes (lm). Central artery (ca); marginal zone (mz); red pulp (rp). × 115.

Fig. 2. A higher power view of the germinal center shown in Figure 1. Spaces containing nuclear debris, the tingible bodies (tb), can be seen. The cells making up the upper half of the germinal center are more compact and appear dark-staining compared to the looser arrangement and lighter staining of cells in the lower half of the germinal center. × 240.



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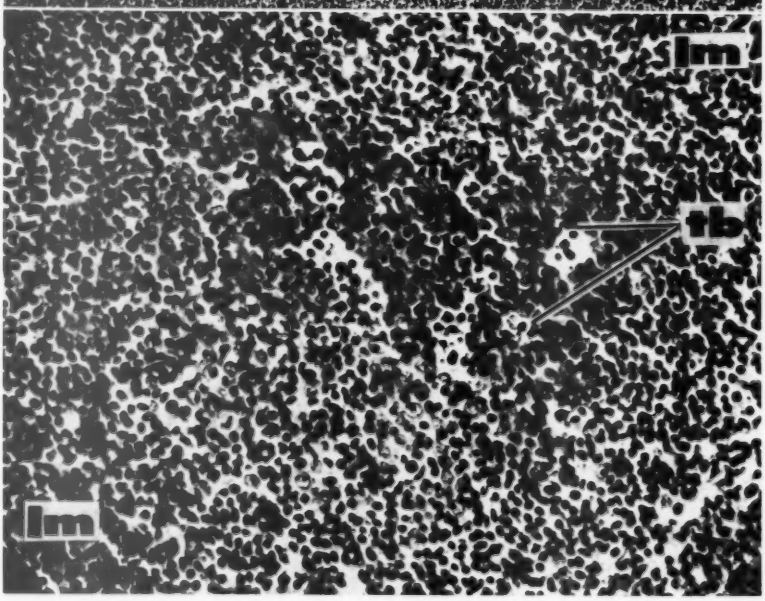

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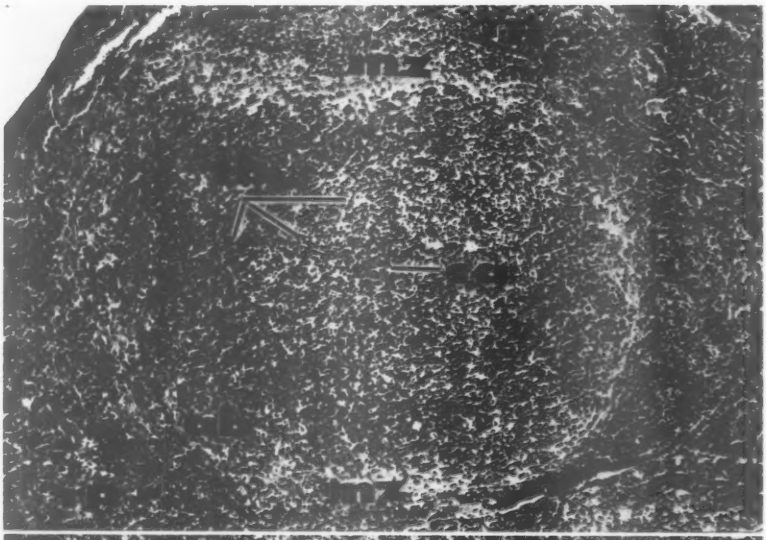

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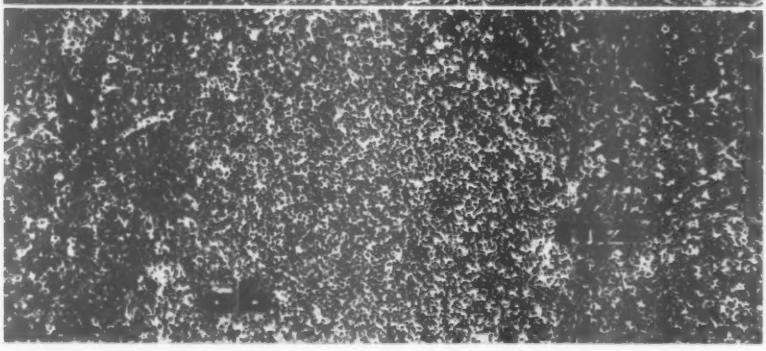


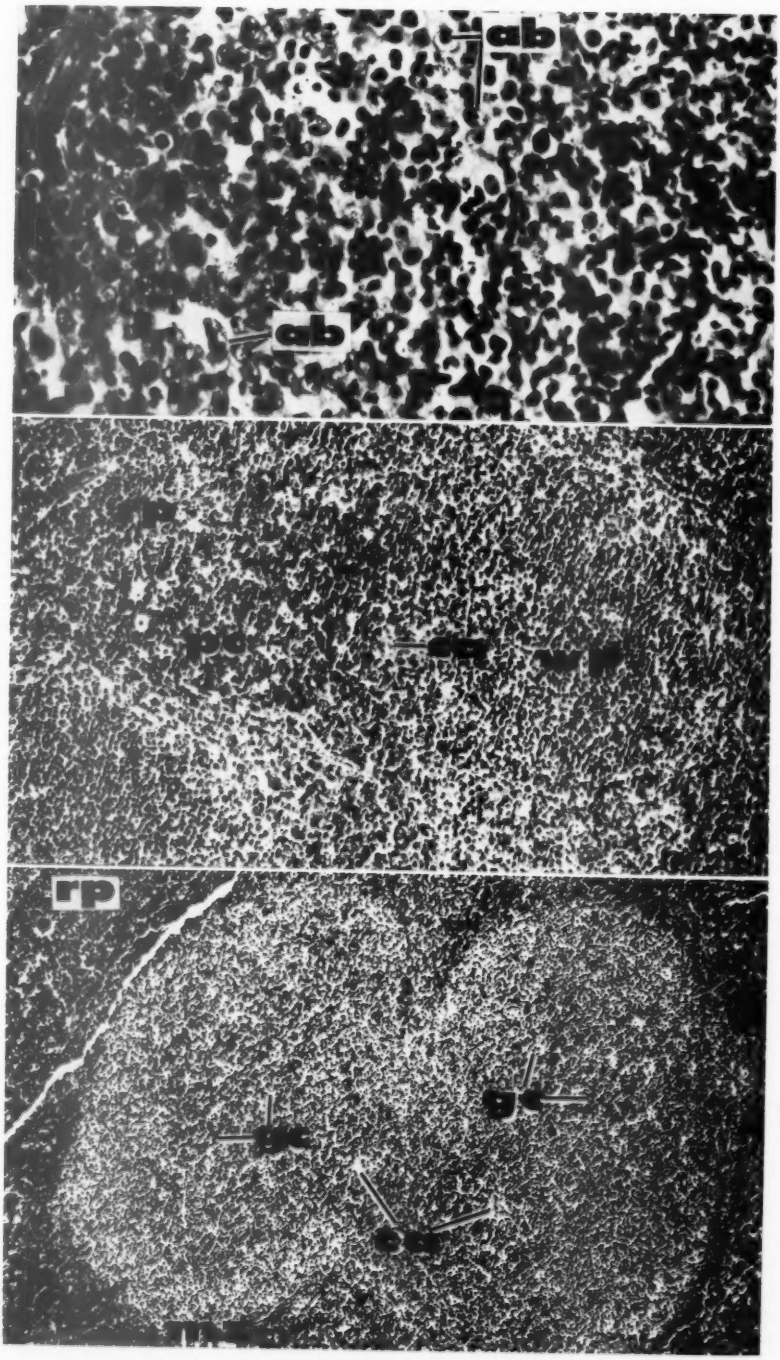
FIG. 3. The white pulp of the spleen 24 hours after the intravenous injection of 1 ml. of 10 per cent sheep red cells. There are swollen marginal zone cells (mz), great reduction in numbers of lymphocytes, absence of germinal centers, an edema-like quality of tissue around the central artery (ca), and many tingible bodies (tb). Proliferation of pale Ab-forming (ab) cells, best seen just inside the marginal zone (mz), also is apparent at this time. × 115.

FIG. 4. White pulp of the spleen 2 days after the injection of 1 ml. of 10 per cent sheep red cells. There is nearly complete loss of normal white pulp architecture, with extensive proliferation of Ab-forming (ab) cells. There is great reduction in the numbers of lymphocytes. × 115.

FIG. 5. Higher power view through the left-hand margin of the white pulp shown in Figure 4. Many pale Ab-forming (ab) cells containing vesicular nuclei are present with some scattered lymphocytes. × 460.

FIG. 6. A mass of plasmacytic cells (pc) continuous with the red pulp (rp) of the spleen, yet appearing to occupy a portion of the white pulp next to the central artery. The remainder of the white pulp (wp) is to the right of the central artery. × 90.

FIG. 7. Splenic white pulp 5 days after sheep red cell injection. Two very large hyperplastic regenerating germinal centers (gc) are evident. × 90.



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APPARENT TRANSFER OF HUMAN HEPATITIS VIRUSES TO DUCKS

H. L. Ratcliffe, Sc.D., and John A. Flick, M.D.

From the Penrose Research Laboratory, Zoological Society of Philadelphia, the Department of Pathology, School of Medicine, and the Department of Microbiology, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa.

Lucké and Ratcliffe, in 1949, reported experiments that strongly suggested the susceptibility of pigeons, ducks, and European starlings to infection by the viruses of infectious hepatitis and of serum hepatitis of man.[1] Subsequent investigations in other laboratories, however, have failed to confirm these observations, or have given doubtful confirmation.[2-6] It seems desirable, therefore, to present the results of additional experiments that support the original suggestion. This study also may aid in explaining some of the unsuccessful experiments of others.

Material and Methods

Pekin ducks were purchased from a commercial hatchery as "day-old" birds and received in the laboratory within 36 hours after hatching. Immediately after arrival the ducks were divided into groups of 15 to 20 for separate compartments, each about 6 square feet, of multiple-unit commercial brooders. Water was available to the birds from this time on, but food was withheld until about 72 hours after hatching. The ducks were fed a high-quality mixed ration (about 25 per cent protein) that supported a rapid rate of growth.

At 2 to 3 weeks of age the birds were transferred to "broiler-batteries," each compartment of which provided about 4 square feet of floor space. An adequate space allowance was found to be about 1 square foot per kg. of body weight. Smaller space allowances interfered with growth and, sometimes, with bone development but, on the other hand, isolated ducks did not grow well either. Weights were recorded twice each week.

This report will be limited to experiments with icterogenic plasma and with a filtrate of pooled feces from patients with infectious hepatitis before, during, and after the onset of jaundice. The sample of plasma, which was designated "plasma pool 310," had been supplied to the late Dr. Balduin Lucké by Dr. Joseph E. Smadel, and the fecal samples by Dr. John R. Neefe. The plasma was received in the frozen state and was held at −20° C. or lower, until used. The stools were frozen separately at −20° until processed.

The fecal filtrate was prepared as follows: Stools were thawed, pooled, and emulsified in physiologic saline in a Waring blender, centrifuged briefly to remove the heavier materials, and diluted in saline to a 20 per cent (by weight) suspension. This suspension was then clarified by centrifugation, passed through a Seitz filter, distributed into vials, and tested for sterility. The stools were processed at 20° C. and the filtrate held at 4° C. until it had been found to be free of viable bacteria; it was then frozen in vials and stored at −20° C. or lower.

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Material for inoculation was thawed rapidly, diluted in saline, and injected within an hour after removal from the dry-ice chest. Inoculums were limited to 0.2 to 0.3 ml. volumes, injected into the jugular vein, which was readily visible after the neck of the duck had been clipped.

Control ducks in the experiments with icterogenic plasma received injections of plasma from an individual who, before and after he had supplied this sample, gave blood for transfusions. The subsequent history of the recipients suggested that he was not a carrier of the virus of serum hepatitis. Control ducks in the experiments with the filtrate of feces were injected with material that had been heated to 70 to 75° C. for one hour.

The infectious agents used in this study were found to spread readily from inoculated to control ducks that were kept in the same room. Chances of accidental transfer were lessened by using separate rooms and attendants. However, accidental transfer was prevented completely only by using separate buildings and attendants.

The ducks were killed by decapitation. Tissues were fixed in 10 per cent neutral formalin within 10 minutes after reflex muscular activity had ended, embedded in paraffin, cut at 5 μ and stained with hematoxylin and phloxine or hematoxylin and eosin.

Observations

Growth Rates as Measures of Transmission

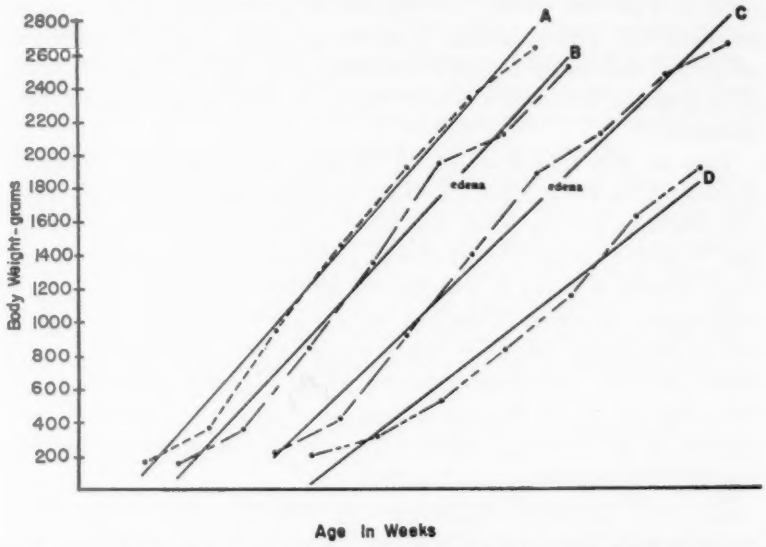
The mean weights of 4 groups of ducks from 3 experiments with material that was assumed to contain the agents of human hepatitis are given in Text-figure 1. These records started one week after hatching, which was the time of inoculation, and, for groups A, B and D, ended with the seventh week. The records of group C were carried through the eighth week.

The solid lines which intersect the graphs of mean weights for each group illustrate regressions of weight on age. These lines were fitted to the data by the method of least squares. The calculated equations for each line (A through D) and the standard error of its slope, are given in the legend. The graphs of the weight records obviously must have had approximately a common origin (about 200 gm. at one week of age). They have been separated in time only to avoid confusion in preparing and reading the chart. Age in weeks is indicated by the points on the graph—one for each week.

Group A contained 25 birds, each of which was injected with normal plasma. The values for this group are representative of control birds that were fed and housed according to the technique outlined in the foregoing section. It is evident from these records that Pekin ducks grow rapidly during their initial 2 months of life. Mean weights increased from less than 200 gm. to more than 2 kg. in 7 weeks.

Group B contained 22 ducks, each of which received an inoculum of plasma pool 310, diluted in an equal volume of saline. The regression coefficient for these records shows that the growth rate for this group was essentially identical with that for group A.

However, the weight records for group B showed a sharp increase during the fifth week, followed by a much smaller gain within the sixth week. This phase of the growth curve was associated with the development of perceptible edema of the feet, legs, and anterior body wall in about two thirds of the birds of this group. Edema disappeared as promptly as it had developed, but it certainly contributed to body weight. Thus, the correspondence between the rates of growth of groups A and



TEXT-FIGURE 1. Growth rates of Pekin ducks through the seventh (eighth, group C) week of age. Records for each group begin at one week, but for convenience the graphs have been separated in time. Group A, control; group B, injected intravenously with icterogenic plasma; group C, inoculated with fecal filtrate from cases of epidemic hepatitis; group D, inoculated with dilute icterogenic plasma (1:50,000). The solid lines which intersect the graphs of weights are fitted to these data by the method of least squares. The calculated equation for each line and the standard error of its slope are as follows:

A: $y = -361 + (440 \pm 17.55)x$ B: $y = -356 + (419 \pm 23.68)x$
C: $y = -184 + (357 \pm 18.77)x$ D: $y = -275 + (300 \pm 22.90)x$

B probably depended more upon the development of edema than upon actual growth.

This experiment with material from plasma pool 310 was repeated twice with other groups of 25 ducks and with the same results, which was surprising since this material, in the same concentration, had induced fatal hepatitis in European starlings.[1] At the same time, accidental induction of hepatitis in control ducks caged in the same animal room suggested that the infectious agent in this material might be much more

active in higher dilution. Hence the experiments that are illustrated by the records of group D.

Group D contained 26 ducks, each of which was inoculated with a sample of plasma pool 310, diluted 1:50,000 in saline. The differences between the growth rate of group D and group A or B are statistically significant ($P<0.001$). Two weeks after inoculation the mean weight of this group was little more than half of that for control group A. This difference decreased after the fifth week and at 7 weeks amounted to about a 30 per cent deficit. The response of a second group of 26 ducks to inoculation with a sample of plasma pool 310, diluted 1:10,000 in saline was essentially identical with that of group D. However, funds did not permit further titration of this or of other suspensions of these materials.

Thus we were limited to testing the fecal filtrate on only one group of ducks. This was group C (Text-fig. 1) which also contained 26 birds. The inoculum for this group was diluted in an equal volume of saline. The weights of group C from weeks 3 through 5 corresponded closely to the weights of group B. This may be attributed in part at least to the development of edema of the feet, legs, and anterior body wall in over half of the group. Edema in this group also was most intense during week 5, then as promptly disappeared, which, of course, was reflected in mean weights and in the rate of gain. In spite of this correspondence to group B, the difference between the growth rates of groups A and C was significant statistically ($P<0.05$).

The foregoing records suggest that an agent or agents of either serum hepatitis or infectious hepatitis usually induced a mild, transient disease in young Pekin ducks. This disease was largely reflected in the growth rate, and recovery was essentially complete within 6 to 7 weeks. For example, the mean weight of group C at 8 weeks and after the edema had disappeared had come to equal that of control group A at 7 weeks. Moreover, at necropsy at 8 weeks, the livers of birds of Group C were within normal limits of size, color, and consistency. Microscopic alterations in these livers were limited to small collections of inflamatory cells (Fig. 5).

The effects of the induced disease, to judge by the weight records, seemed to have been more prolonged in group D, but at necropsy of these birds at 7 weeks, evidence of continued hepatic disease was no more convincing than in the ducks of group C. However, signs of disease (changes in color and consistency of the livers) were demonstrated readily in birds killed from 2 to 4 weeks after inoculation, and 3 to 5 weeks of age.

Tissue Changes

The livers of normal Pekin ducks (controls) at 3 to 7 weeks of age were highly uniform in color and consistency: translucent red-brown and firm (Fig. 1A). Cut surfaces of these organs were always clear and sharp.

In contrast, the livers of ducks killed for study 2 to 4 weeks after inoculation frequently were soft and relatively friable, their margins rounded, and the capsular and cut surfaces turbid (Fig. 1B). These softer, swollen livers also appeared in a considerable range of colors, dull yellow-brown to reddish or grayish purple. These evidences of disease were found to be most frequent about 3 weeks after inoculation, but never in more than half of any group killed at this interval. Before and after week 3 the frequency of clearly evident changes was distinctly less. None was recognized 6 or more weeks after inoculation.

The microscopic appearances of the livers of normal young Pekin ducks were as highly uniform as their macroscopic features. Usually the vascular bed of these livers was essentially empty (a result of exsanguination) and lobular outlines inconspicuous. The hepatic cells appeared to be relatively regular in size, their outline clearly defined, cytoplasm pale-staining and lace-like, and nuclei relatively large and hyperchromatic. As a rule, hepatic cells seemed to be arranged into layers two cells in width between sinusoids (Fig. 2).

The earliest recognizable histologic evidence of hepatic disease in these ducks was a loss of this highly uniform structure and apparent collapse of the vascular bed. Livers that were believed to represent the earlier phases of this change (1 to 2 weeks post inoculation) were composed of cells that ranged widely in size and shape and in the staining qualities of cytoplasm and nuclei. Many cells in sections of these livers appeared as almost empty spaces enclosed by heavy walls from which projected strands of cytoplasm and distorted nuclei. Intervening cells often were indistinctly outlined, their cytoplasm increased irregularly in staining density, and their nuclei ranged from large and hyperchromatic to distorted and pale (Fig. 3). Later stages of this process (2 to 3 weeks post inoculation) were characterized by a reduction in the numbers of the heavily walled, clear-staining cells, an increasing disappearance of cell outlines elsewhere in the liver sections, a greater density in the staining quality of the cytoplasm, especially in these apparently syncytial foci, and a greater range in nuclear size and staining qualities.

Thus, in some instances the livers of ducks killed for study 2 to 4 weeks after inoculation contained large foci of apparently fused cells (hepatic syncytium?) subdivided by sinusoidal capillaries and arranged into the usual lobules. Occasionally one of these foci contained numbers

of dividing nuclei, some of which seemed to have undergone abnormal mitosis (Fig. 4). These suggestions of hepatic syncytium with densely staining cytoplasm and numbers of dividing nuclei were not encountered in sections of the livers of ducks killed more than 4 weeks after inoculation. Between weeks 5 and 10 after inoculation, the hepatic cells seemed to be returning more and more to the pale-staining, lace-like cytoplasm characteristic of the normal ducks. During the earlier phases of this change (recovery?), occasional clear-staining cells still were found, but a considerable majority approached the normal appearance. They differed chiefly in their range in size and in the relatively large size of their nuclei. At the same time Kupffer cells were increased throughout the vascular bed, and appreciable numbers of monocytes had accumulated about the portal spaces (Fig. 5).

Discussion

The foregoing account of the morphologic alterations in the liver after the inoculation of young ducks with materials presumably containing the viruses of human hepatitis has, of necessity, omitted many of the sequential stages in the initiation, development, and regression of the response to infection. A more complete description would, however, have required much larger experimental groups, which probably would not have contributed essential information.

In any event, these experiments demonstrate that young ducks responded to inoculation with materials that presumably contained the viruses of human hepatitis by developing a relatively mild, acute, nonsuppurative hepatitis. The possibility that this response reflected the activation of a latent virus cannot be ignored. However, the virus hepatitis of ducks endemic in the region from which our experimental birds were purchased could be excluded. All phases of the disease induced in these experiments—incubation period, clinical signs, duration, mortality, and lesions—were distinctly different.[7-9] Thus, the present observations support the earlier claim of Lucké and Ratcliffe, who reported that the same or equivalent materials caused a more severe form of hepatitis in starlings and pigeons.[1] The present experiments also have shown that in ducks, icterogenic plasma was most active in high dilution, which suggests that this virus may exhibit the interference phenomenon in more concentrated suspensions.

The results of these experiments also are contrary to a majority opinion.[3,4] Reviews of attempts to transmit the viruses of serum hepatitis and infectious hepatitis of man to experimental hosts other than man list many failures, especially when mammals have been the subjects. Success with birds has been reported more often.[2] The few experiments

reported here have been drawn from a much larger experience. This experience leads us to suggest that many recent failures to confirm the infectivity for birds of the viruses of human hepatitis may be attributed to inadequate methods, to unwise choice of experimental subjects, or to a combination of these factors. For example, the hepatic disease induced in the ducks in this study had an incubation period of less than 3 weeks, after which not more than one half or, at most, two thirds of a group developed recognizable lesions at any time. Then too, the disease apparently remained active for only about 2 weeks, after which recovery was rapid and complete. Deaths from hepatitis did not occur in any of the experimental groups of ducks maintained under the conditions described herein. In fact, the only deaths from hepatitis that occurred in the course of this and earlier work were among European starlings (*Sturnus vulgarus*).[1] These birds were from a group of 60 that had been inoculated intravenously with icterogenic plasma. Only 10 of this group developed recognizable weakness within 3 to 4 weeks, and 3 died of acute hepatic disease. It seems obvious, therefore, that brief, cursory attempts at transmitting the hepatitis viruses of man to small groups of birds of uncertain age and history are completely meaningless.

It should be emphasized, too, that both the macroscopic and microscopic alterations that have been described and illustrated here must be regarded as nonspecific. That is, corresponding lesions might be induced by a variety of agents, or perhaps by poisons, or deficiencies. The techniques used in these experiments apparently excluded these extraneous factors.

Summary

Human plasma known to contain the virus of "serum hepatitis," and a bacteria-free filtrate of feces that presumably contained the virus of infectious hepatitis of man were inoculated into domestic ducks at one week of age. The plasma in high dilution and the undiluted fecal filtrate caused statistically significant reductions in the growth rate of these birds. This change began within 3 weeks of inoculation and continued through the following period of 3 weeks. It was associated with the development of acute, nonsuppurative hepatitis from which recovery was rapid and complete.

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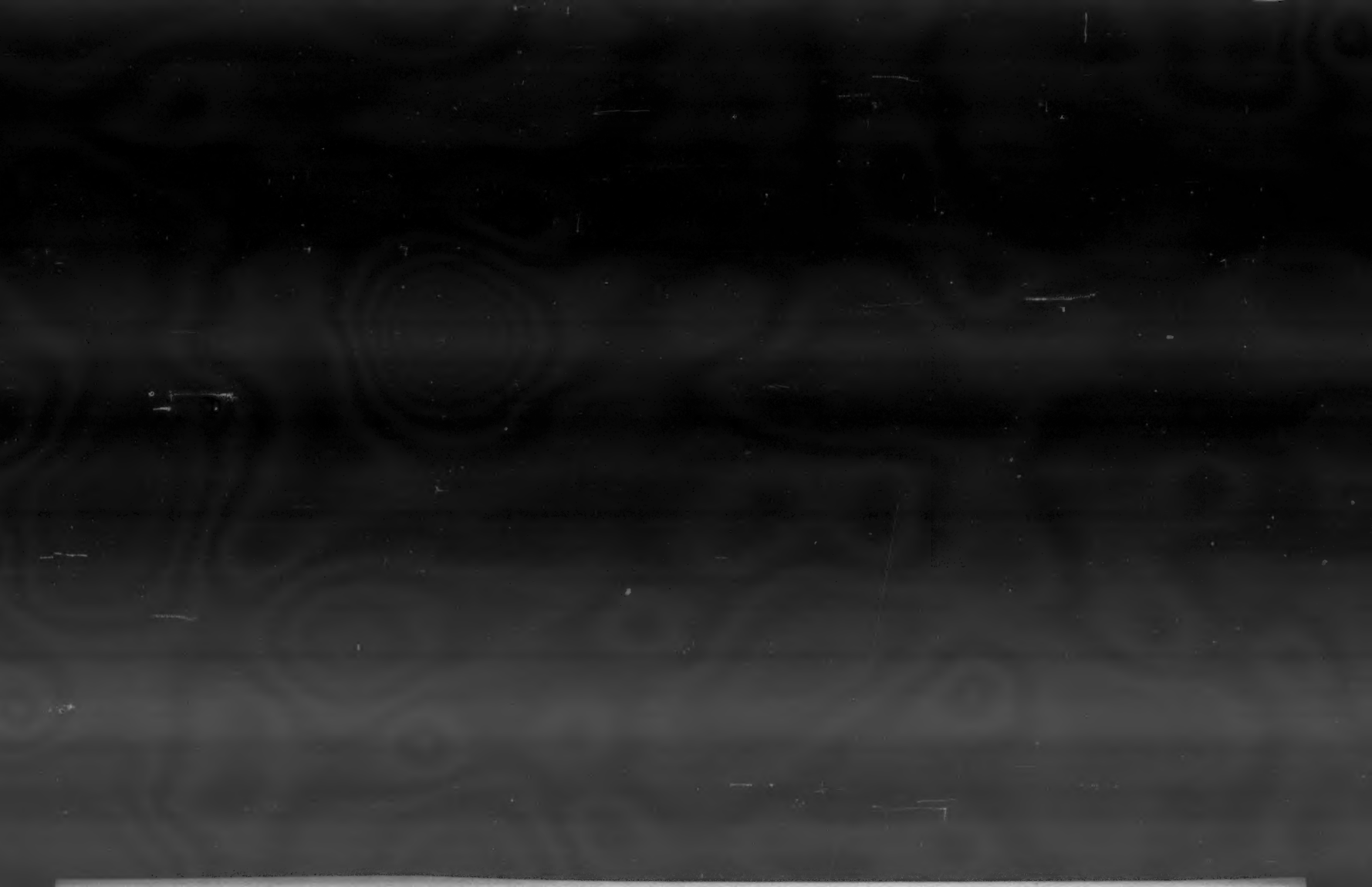
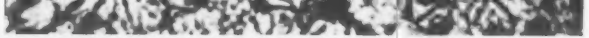
LEGENDS FOR FIGURES

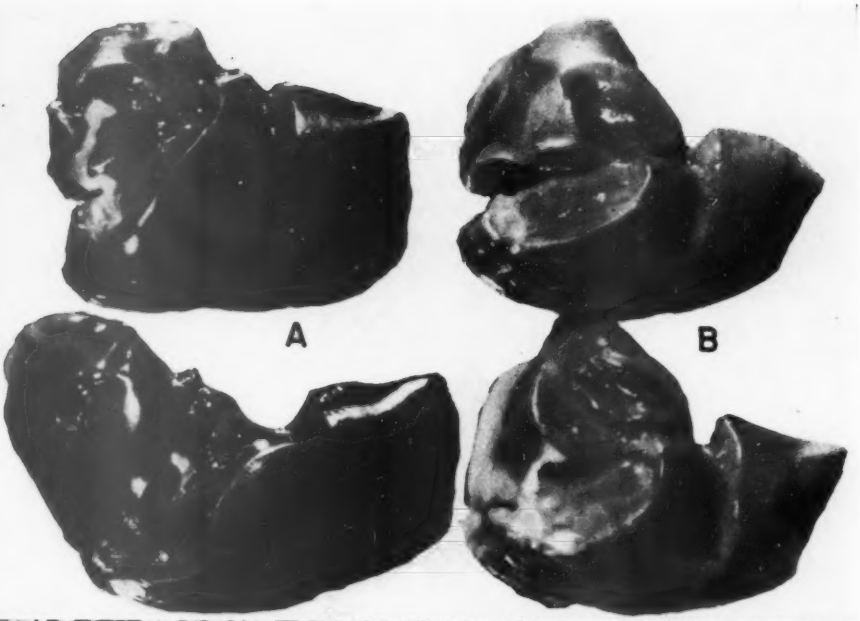
Photomicrographs were prepared from sections stained with hematoxylin and eosin.

FIG. 1. Macroscopic appearances of the livers in normal young, growing domestic ducks (control A) compared to those in young ducks of a corresponding age, two weeks after inoculation with bacteria-free filtrate presumably containing the virus of epidemic hepatitis (B). These specimens were photographed within 15 minutes after death. The anterior surfaces are shown with the free edges to the viewer's right; the groove between the lobes accommodates the heart. The cuts in the right lobe were made in taking samples for histologic examination. Differences in color and consistency are obvious and reflect a reaction of young ducks to the viruses of human hepatitis.

FIG 2. The usual histologic pattern in the liver of young growing ducks used as controls in this study. The liver cells and their nuclei are highly uniform in size and internal structure and tend to an acinar arrangement. × 700.







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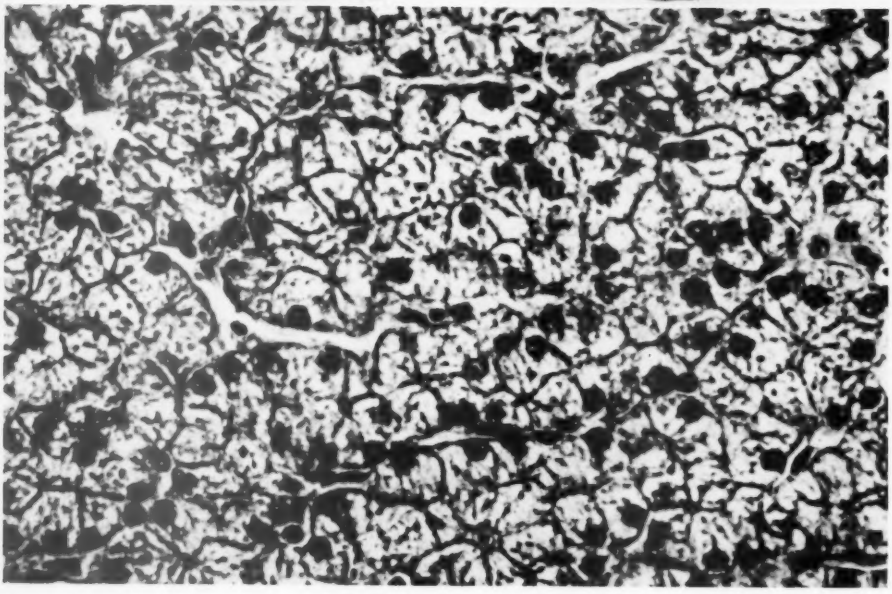

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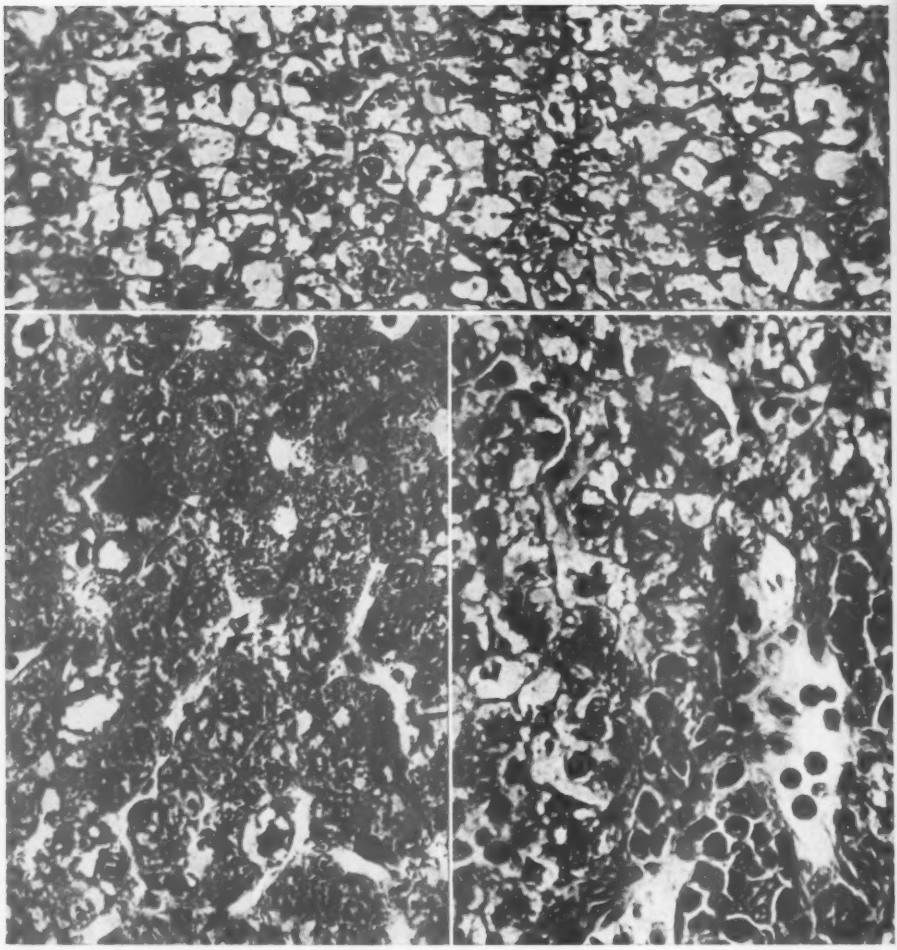
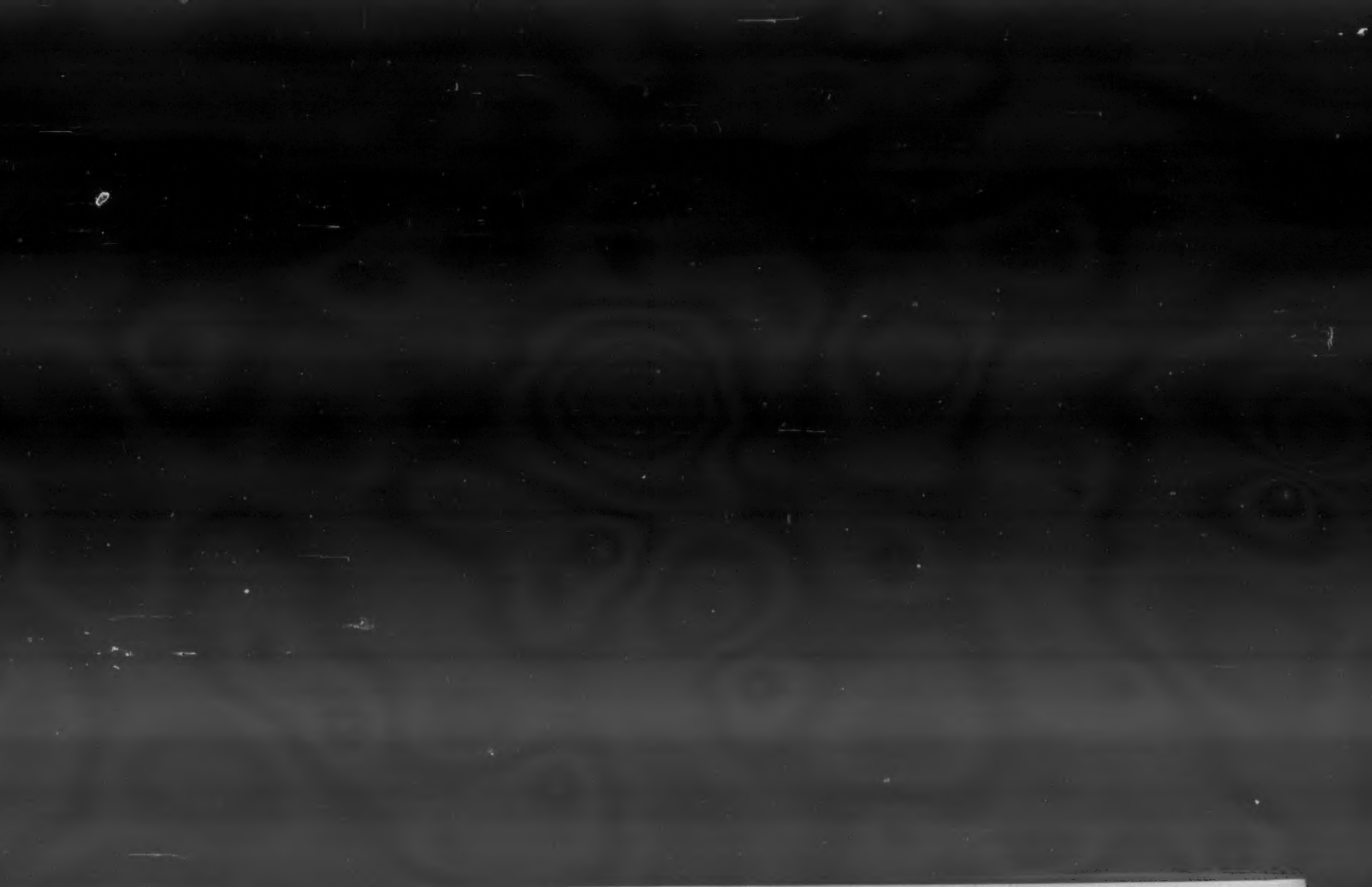


FIG. 3. The histologic appearances of the liver in a young duck 3 weeks after inoculation with a fecal filtrate. Vascular channels are indistinct, and hepatic cells show an increased size range. Many cells appear as clear-staining (or empty) spaces with heavily stained walls. In other foci the cytoplasm stains more heavily than in the controls, and here cell outlines are indistinct. Nuclei of hepatic cells range widely in size and staining qualities. This liver was dull, pale reddish yellow, swollen and soft. × 700.

FIG. 4. A recovery (?) stage in the liver of a young duck after inoculation with either of the viruses of human hepatitis. Compare with Figures 2 and 3, and note the nuclei in division (black arrows), the poorly defined cell outlines, and the range in the size and staining qualities of the nuclei. This stage apparently was relatively brief. This bird was killed 17 days after inoculation. × 700.

FIG. 5. The common histologic appearance of the liver in young growing ducks 7 or more weeks after inoculation when effects upon growth had ceased. Cells are less uniform and more often bi-nucleate than in control birds of the same age. Kupffer cells are increased in number, and monocytes have collected about a portal space. Compare with Figure 2. × 700.



FACTORS INVOLVED IN RECOVERY FROM EXPERIMENTAL SKELETAL MUSCLE ISCHEMIA PRODUCED IN DOGS

I. Histologic and Histochemical Pattern of Ischemic Muscle

Robert E. Scully, M.D.; James M. Shannon, M.D., and G. Richard Dickersin, M.D.

From the Departments of Pathology and Surgery, Harvard Medical School, and the James Homer Wright Pathology Laboratories and the Department of Surgery, Massachusetts General Hospital, Boston, Mass.

Failure of human skeletal muscle to recover from an acute episode of ischemia caused by interruption of its arterial blood flow may necessitate surgical excision of necrotic tissue or even amputation of an extremity. Both experimental investigation [1] and clinical experience [2] have shown that as the duration of ischemia is prolonged, recovery is less likely, in spite of restoration of circulation. For this reason, during the Korean conflict, attempts were made to reduce the time interval between trauma and reparative surgery in order to increase the survival rate. Little consideration, however, has been given to a number of other variables that might influence recovery. The present series of experiments was undertaken in order to evaluate some of these in the hope that the conclusions might prove helpful to the surgeon called upon to perform restorative vascular surgery on a limb that is hovering between viability and death. The present communication describes the technique utilized in producing skeletal muscle ischemia, and presents results that will serve as a base line for later experiments. In these, the degree of restoration of circulation, the temperature of the muscle, and the coagulability of the blood will be altered in attempts to improve recovery.

Review of the Literature

Several experimental approaches have been used to produce skeletal muscle ischemia. In small animals, application of a tourniquet has proven effective.[3] In larger animals, the problem has been more difficult. Brooks [4] found that only by ligating multiple terminal branches of the aorta, was he able to bring about muscle necrosis in the hind limb of the dog. Simple ligation of a single large peripheral artery was ineffective because of the existence of rich collateral circulation. By ligating the femoral artery, transecting the remaining soft tissue of the thigh, and leaving the limb attached only by the femur, Miller and Welch [1] were able to

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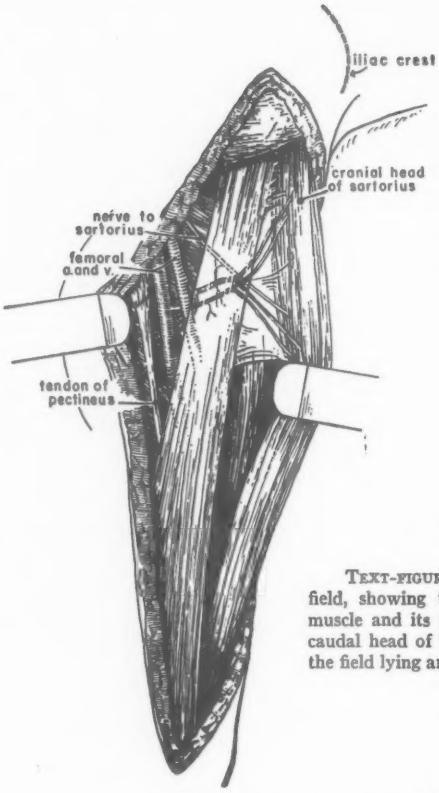
produce gangrene in 80 per cent of dogs within 24 to 30 hours. Apparently the osseous arteries were large enough in the remaining animals to supply the entire extremity with blood sufficient for survival; after an ischemic interval of only 1 to 6 hours prior to restoration of circulation through the femoral artery, 90 per cent of limbs survived. The salvage rate fell to 50 per cent after ischemia of 12 to 18 hours' duration.



TEXT-FIGURE 1. Drawing of the operative field, showing the cranial head of the sartorius muscle and its vascular and nervous supply. The caudal head of the muscle is seen in the center of the field lying anterior to the vascular pedicle.

EXPERIMENTAL METHODS

Twenty-two mongrel dogs, weighing between 11 and 21 kg., were anesthetized by intravenously administered Nembutal® (0.6 ml. per kg.). Under sterile operating conditions, the cranial head of the sartorius muscle was exposed through a vertical incision on the anteromedial aspect of the thigh, extending from the anterior superior spine of the ilium to the patella (Text-fig. 1). It was then dissected free from origin to insertion except for an attachment by a neurovascular pedicle, which entered it at the junction of the upper and middle thirds on the medial aspect. Division of the caudal head of the muscle, which lay anteriorly, exposed the pedicle in its entirety. Usually, the blood supply to the cranial head was carried by a single large artery. However, in order to eliminate the possibility of additional circulation of significant degree, the nerve, vein and artery in the pedicle were carefully dissected for 1 to 2 cm.

proximal to the muscle, and all small vessels that were encountered were ligated. The main artery was then dissected free to its point of origin and all branches were ligated; the main artery usually arose from a large penetrating branch of the femoral artery or from the femoral artery itself immediately adjacent to this branch. Kocher clamps were then placed on the proximal and distal ends of the muscle, and these were transected, interrupting all vessels entering at the origin and insertion. These vessels were small, but on the several occasions when they had not been divided, they furnished sufficient blood to the muscle to prevent the development of recognizable ischemia. The muscle ends were then resutured at their original points of attachment so that the length of the muscle approximated the normal. In several experiments resuturing was not done; the muscle was allowed to contract, in order to contrast the ischemic alterations in shortened muscle with that extended to rest length. The final step in the procedure was ligation in continuity of the femoral artery above and below the branch to the cranial head of the sartorius. The large penetrating branch of the femoral artery was also ligated distal to the origin of the main artery to the muscle.

Complete interruption of the arterial inflow to the muscle was regularly achieved. The anatomic variations were infrequent and never of significant magnitude. Occasionally there were single accessory muscle arteries, but these always arose within 1 to 2 cm. of the main artery and it was possible to isolate them in a similar manner and to control their blood flow by placing the ligatures on the femoral artery above and below them as well as on the main arteries.

At the termination of the procedure, 300,000 units of penicillin and 0.5 gm. of streptomycin were administered intramuscularly in one of the forelimbs.

At regular intervals following interruption of blood flow, the responses of the muscle to mechanical and electrical stimulation were tested. Mechanical stimulation consisted of pinching the muscle and the nerve in the pedicle with a smooth forceps. Electrical stimulation involved application to the nerve and the muscle of a faradic current with varying grades of intensity provided by a Bovie unit. At regular intervals after ligation of the femoral artery, the neurovascular pedicle was clamped and samples of the ischemic muscle were obtained for microscopic study. In the earlier experiments all areas of the muscle were examined. It was soon discovered, however, that the portion of muscle midway between the pedicle and the lower pole was freest of artifact and revealed the most consistent alterations. The superior and inferior portions and the region adjacent to the pedicle were apt to show hemorrhage and lesions thought to be related to the trauma of dissection rather than to ischemia *per se*. Therefore, in later experiments changes in the mid-portion of the muscle were investigated in greatest detail. Longitudinal and cross-sectional blocks of muscle were placed in 10 per cent formalin and carried through paraffin for staining by hematoxylin and eosin, and the periodic acid-Schiff (PAS) method. Frozen sections of formalin-fixed tissue were stained with Sudan IV for neutral fats. In certain cases, fresh frozen tissue was used to demonstrate succinic dehydrogenase according to the method of Seligman and Rutenburg.[8]

Because of the differences in content of muscle constituents such as glycogen and fat that exist from one animal to another in the absence of ischemia, samples of normal muscle were taken as controls in each experiment. In the early phases of the project, the control specimen was obtained from the same leg as the experimental muscle. Although there was no detectable evidence of damage in these specimens, it was subsequently considered preferable to sample a muscle from the forelimb as a control.

Observations

Physiologic Features

Muscles totally ischemic for 3 or more hours failed to contract on direct electrical and mechanical stimulation and on electrical and me-

chanical stimulation of their nerves. No electrical tests of contraction were done after shorter intervals of ischemia, although mechanical stimulation of the muscle and its nerve one hour after the onset of ischemia failed to elicit a response.

Gross Abnormalities

The control muscles were of normal brownish-red color and of moderately firm consistency. With advancing ischemia, the experimental muscles became increasingly pale, pinkish yellow, soft and flabby.

Microscopic Features

Control Muscle. The control muscle in hematoxylin and eosin preparations was composed of fibers in close approximation to one another. The pattern of cross striation varied with the method of fixation. When a sample was cut from the muscle and placed directly in formalin, the pattern was the typical one of muscle in the shortened state[6] (Fig. 1). The most prominent transverse eosinophilic bands were the isotropic contraction bands located in the position of the Z lines, but appearing coarser due to an addition on either side of eosinophilic material that may have migrated from the A bands.[6] Between the contraction bands were broader, pale, anisotropic bands interpreted by Jordan as exposed H discs[6]; a second variety of eosinophilic band, more delicate than the contraction band and believed to be the M line, was often discernible bisecting the H disc. The longitudinal fibrils were generally wavy, but appeared straight in a proportion of fibers that varied from one specimen to another. When, on the other hand, the muscle specimen was maintained at rest length on a wooden slab during excision and fixation, the pattern of cross striation differed from that of the shortened muscle (Fig. 2). Now the A band appeared as a prominent eosinophilic bar with a central pale zone corresponding to the H disc; the M line was no longer visible. Between the A bands were broad pale I bands bisected by delicate Z lines. The longitudinal fibrils were straight.

On cross section the fibers had a fine needle-point granulation. The muscle nuclei were predominantly oval or elongated, occasionally round; their chromatin was fine and sandlike or coarse and speckled.

Sections stained by the PAS method for glycogen, with diastase digestion for control purposes, revealed a considerable variation in the content and distribution of stainable material from muscle to muscle and to some extent from area to area in the same muscle. Most commonly the glycogen appeared as irregular, sharply defined, deeply staining patches occupying most of the width of the fibers. These patches were either granular or dense and amorphous, and they characteristically occurred

adjacent to areas devoid of stainable material. Instead of forming wide patches, the staining was often intense for a considerable distance along the periphery of the fibers. On cross section, corresponding patterns of distribution consisted of diffuse granular or amorphous staining of the entire fiber, or intense staining at one edge of the fiber, giving the appearance of a dark cap. The irregularities in distribution may be partly artifactual; if cold fixation is employed prior to staining for glycogen, its distribution is more even throughout the cell.[7] In fibers containing glycogen in moderate amounts, the granules were arranged primarily along the contraction bands; however, some fibers showed a more diffuse scattering of granules and larger globules without relation to specific bands. The saliva-digested control sections showed little or no substance with strong affinity for the stain; the sarcolemma, contraction bands, A bands, Z lines and M lines, however, remained weakly PAS-positive.

The amount of fat, as revealed by the Sudan IV stain, varied in amount from little or none to moderate. The distribution of droplets within an individual muscle was also subject to considerable variation; a single fiber might contain large amounts while adjacent ones had none. Although often a fiber contained a uniform quantity of fat for as great a distance as it could be followed in a longitudinal section, at other times the fat was concentrated in short patches that faded gradually into fat-free portions. Segments of fibers containing moderate to large quantities generally presented a uniform dissemination of droplets; in fibers with smaller amounts, the distribution was irregular. On high power examination, most of the fat droplets were fine and somewhat irregular in shape. When small, they appeared to lie at the junction of the I and A bands, protruding more into the former than the latter; as they increased in size they occupied more and more of the I band, the A band remaining relatively free.

Stains for succinic dehydrogenase showed a uniform diffuse distribution of finely granular material in the fibers. The granules varied slightly in size in most specimens; in some they were coarse and irregular. In longitudinal sections, the granules appeared to be distributed along the A bands.

Ischemic Muscle, Resutured. There was some variation from animal to animal in the length of time the muscle was capable of withstanding total deprivation of blood. The temporal aspects of the structural alterations to be described are those of an average specimen. The samples of ischemic muscle were placed directly in fixative with no attempt to preserve them at rest length.

After 2 hours no abnormality was detectable except for some reduction in the waviness of fibrils; the striation pattern of shortened muscle was

maintained. After 3 to 4 hours of ischemia almost all the fibers were composed of straight fibrils. Most of them now showed the striation pattern of rest-length muscle, with coarse eosinophilic anisotropic bands alternating with pale isotropic bands bisected by delicate Z lines. With the change of cross-striational pattern, the longitudinal fibrils were now less distinct than in the shortened control muscle. In some of the fibers, neither the A nor I bands retained their perpendicular orientation to the sarcolemma; they now appeared as arcs of varying degrees. Occasional fibers were partially or wholly detached from their endomysial sheaths; this change was most apparent on cross section.

In the next 2 hours coarsening and curving of the A bands and separation from the endomysium became more widespread. Meanwhile, distinctive alterations began to appear at the periphery of the muscle (Figs. 3 and 4). Two layers of variable width could now be identified—an outer one, generally a few fibers in thickness, of normal muscle cells with a striation pattern similar to that of the shortened control muscle, and within this, a second layer of comparable width made up of fibers showing hyaline swelling of the cytoplasm with loss of structural detail and pyknosis of nuclei. Considerable separation of the swollen fibers and capillary congestion were features of the inner layer.

After 8 to 12 hours of ischemia the changes had become marked and universal in the center of the muscle, which now appeared as an array of parallel elongated isolated fibers. Abnormalities were now, for the first time, clearly detectable in the blood vessels. The capillaries had the appearance of being empty as the result of marked fading of the enclosed red cells. The occasional large artery visualized was necrotic, its smooth muscle fibers having lost their close approximation and appearing as individual units. Now the layering at the periphery of the muscle presented additional features (Fig. 5). The outer zone of normal fibers showed patchy necrosis; the inner layer of hyaline swelling and, to a greater extent, the peripheral rim of the central necrotic zone were massively infiltrated with neutrophils, which formed a layer approximately 0.5 cm. in thickness, sharply demarcated from the innermost necrotic zone. The leukocytes showed very little tendency to penetrate within individual dead fibers.

During the next 13 hours there was very little change in the appearance of the muscle. It is worthy of comment, however, that even at an ischemic interval of 24 hours, the coarse A bands remained distinct. A paling in their centers, corresponding to the H disc, was often discernible, and Z lines were commonly seen as sharp membranes bisecting the I bands (Figs. 6). A second remarkable feature was the absence of clearly detectable abnormalities in the nuclei of the dead muscle cells, although

a greater number of them appeared dark and contained coarse chromatin than in the control muscle. At the periphery of the muscle after 24 hours of ischemia, the layer of neutrophils separated the fibers to a greater extent and was somewhat broader, but showed little additional centripetal extension. The infiltrate, however, had now spread to the outermost layer of formerly viable fibers, which was almost totally necrotic, and communicated with a sea of neutrophils in the perimuscular connective tissue.

After 48 hours the discoid pattern of the fibers was even more striking, with greater and more diffuse staining of the A bands and paling of the I bands. By now some of the nuclei had disappeared; those that remained were almost all pyknotic although occasional normal-appearing forms could still be identified. Some fibers continued to show discernible H discs and Z lines although in general these were much more difficult to make out than in earlier specimens. There was occasional cracking of the fibers between the A bands, so that no more than an empty space could be seen separating them (discoid necrosis) (Fig. 7). At the periphery, the leukocytes showed much karyorrhexis; still there was almost no penetration of individual fibers.

A few muscles examined after 48 hours of ischemia showed accumulations of bacteria morphologically consistent with Clostridia. In these specimens the fibers were extensively fragmented and in a state of disarray; in some areas they had disintegrated into granular debris, the muscle nuclei had almost totally disappeared, and in the zones of most severe degeneration the fibers had lost their birefringence.

Shortened Ischemic Muscle. The 4 muscles that had been rendered ischemic without resuturing their tendinous ends showed a pattern of degeneration that differed from the discoid variety described above. In these specimens most of the necrotic fibers retained the shortened pattern of cross striation but in an exaggerated form. These fibers appeared distinctly wider than normal shortened fibers, showing delicate contraction bands alternating with broader light bands; no M line could be made out in them (contraction necrosis) (Figs. 8 and 9). There was minimal curving of striations, and many of the fibers retained the wavy pattern of longitudinal fibrils, which appeared more distinct than in the resutured muscles (Fig. 8). This delicate pattern of cross striation was still apparent after 48 hours of ischemia (Fig. 9). The separation of the fibers from their endomysial sheaths and the nuclear, vascular, and marginal layering changes were, on the other hand, similar to those occurring in ischemic muscles that had been resutured at their tendinous ends.

Histochemistry of Ischemic Muscle. Glycogen disappeared gradually from the ischemic muscles; however, considerable quantities were still detectable in 3 of 4 specimens examined after an interval of 5 to 6 hours.

As in normal muscle at rest length, most of the remaining granules were localized in the I bands. After 8 to 11 hours of ischemia, glycogen was absent from the fibers occupying the central necrotic zone and the zone of neutrophil infiltration, but was still present wherever viable fibers could be seen at the periphery.

Fat droplets tended to increase slightly in amount in the early hours of ischemia, but later there was considerable quantitative variation. For example, at 8 to 11 hours while there was a moderate increase in 2 specimens, there was a slight diminution in a third. At 22 to 24 hours, there was no discernible increase in the amount of fat in the ischemic muscle over that in the control in 8 of 11 cases; however, a slight increase was noted in 1, and a moderate increase in 2 other specimens. The distribution of the fat primarily in the I bands did not differ from that in the control muscles. There was no predilection of the lipid droplets for any type of fiber—some discoid fibers containing large quantities; others, little or none.

In general, the ischemic muscles showed some decrease in the amount of succinic dehydrogenase as well as a tendency for it to be present in larger and more irregularly sized droplets. However, considerable quantities of positive substance remained in muscle that had been totally ischemic for as long as 48 hours and often it was impossible to distinguish normal from obviously necrotic muscle on the basis of the amount or distribution of the stain.

Discussion

The changes occurring in skeletal muscle that has been totally deprived of its arterial blood supply may be divided into two components for purposes of discussion: (a) an alteration in the pattern of cross striation, and (b) autolysis.

The change in the pattern of cross striation in the resutured ischemic muscle appears to be a direct result of the loss of contractility of fibers maintained at rest length. It is neither a specific nor an invariable effect of ischemia *per se*. When normal muscle is excised at operation or soon after death and is placed in fixative, it shortens. Hematoxylin and eosin stained sections reveal a pattern of striation characterized by a narrow eosinophilic contraction band and a broader, weakly staining, anisotropic band bisected by a delicate line in the region of the M line (Fig. 1). On the other hand, the pattern seen in the ischemic muscle processed in an identical fashion consists of weakly eosinophilic isotropic bands bisected by prominent Z lines and alternating with strongly eosinophilic anisotropic bands (Fig. 6). This pattern is indistinguishable from that of live muscle maintained at rest length during excision and placement in fixa-

tive (Fig. 2), and from that of postmortem muscle that has remained at rest length and has not been excised and fixed until it has lost its contractility as a result of postmortem autolysis. That this change in cross striation pattern in ischemic muscle is not an invariable consequence of ischemic autolysis *per se* is proved by allowing the muscle to shorten by cutting its tendinous ends prior to obstructing its arterial circulation. This results in the development of a much different pattern, one of exaggerated shortening (Figs. 8 and 9). Under such conditions, the fibers, which undergo other changes of a nature similar to those of ischemic rest-length muscle, are widened and show alternating pale anisotropic bands and delicate contraction bands. Moreover, many of the fibers retain the distinct and wavy appearance of fibrils characteristic of the contraction pattern of striated muscle. Dissolution of the I band is not apparent, and no separation occurs in the region of the contraction band.

It is of comparative interest that the necrotic muscle observed in human limbs following acute arterial obstruction may show either a rest-length pattern of striation (Fig. 10) or one of exaggerated shortening (Fig. 11). It is logical to assume that these patterns reflect a loss of contractility on the one hand and a state of contracture on the other, as the muscle fiber undergoes necrosis; the correlation, however, is at present only speculative.

While the basic striation pattern of discoid necrosis is determined by a loss of contractility in muscle maintained at rest length, the fully developed lesion, which is characterized by a series of broad, deeply staining discs alternating with clear spaces, is obviously a consequence of contributory autolytic changes which result in eventual dissolution of the I bands. Moreover, the curving of the discs, while to some extent attributable to absence of shortening of the fibers when placed in fixative, is probably largely an autolytic phenomenon.

The autolytic alterations in skeletal muscle totally deprived of its arterial blood supply are manifested very slowly when examined with the light microscope. Indeed, a striation pattern in which A, I, H and Z bands can be recognized, and a nuclear structure that is hardly distinguishable from that of normal muscle cells characterize a muscle totally ischemic for 24 hours. Moreover, in cases of Volkmann's contracture in human subjects, we and others [8,9] have been able to recognize striation patterns of both shortened and rest-length muscle in fibers assumed to have been dead for 2 to 11 months (Figs. 10 and 11). This extraordinarily slow rate of autolysis of skeletal muscle has been recognized for a long time. Wells [10] in 1906, studying autolysis of tissues *in vitro*, stated: "Muscle tissue undergoes changes very slowly, especially in its cytoplasmic structures. In the myocardium the nuclei fade from the fourth

to the tenth day, but the striations are still distinct after thirty days; striated muscle undergoes changes still more slowly." In 1924, Chen and Bradley,[11] approaching the problem from a chemical viewpoint, concluded that the structural proteins of muscle were resistant to autolysis by intracellular proteases and that their digestion either proceeded very slowly or required the action of enzymes other than those present in muscle.

Autolysis of skeletal muscle in the living animal progresses more rapidly than *in vitro* for two possible reasons: (a) the temperature at which autolytic changes occur most rapidly is 37° C., the approximate body temperature,[12] and (b) bacterial infection may accelerate autolysis. A pertinent fact that requires consideration in the present experiment is that Clostridia either normally reside in skeletal muscle of dogs or are introduced readily from the skin surface.[13] Although cultures of the ischemic muscles were not taken, organisms resembling Clostridia did appear in tissue sections in 2 muscles ischemic for 48 hours; these specimens showed the most advanced degrees of autolytic change. It is conceivable that organisms too few to recognize in tissue sections contributed to the autolysis in the less advanced cases. However, the failure to find them in numerous microscopic sections of all but 2 specimens and the chemotherapy administered to the animals minimize this possibility.

The alterations observed at the margin of the necrotic muscle are similar to those described surrounding infarcts of other organs, both experimental and human.[14–16] They are of interest not only in respect to structural manifestations of cell death in general, but more specifically insofar as they contribute to an understanding of the pathogenesis of hyaline necrosis (waxy, Zenker's) in contrast to the striate forms (discoid and contraction) of muscle necrosis. The marginal changes in the present experiment were artifactual in that they depended on contact of the ischemic tissue with surrounding viable tissue, and upon diffusion of metabolites into and out of the former. The peripheral layer of totally ischemic tissue survived, at least in the early stages of the experiment, appearing entirely normal histologically and histochemically. Deep to this layer were fibers that showed swelling and hyalinization of cytoplasm with obliteration of the striate pattern and nuclear pyknosis (Zenker's necrosis). These fibers, in other words, were not adequately exposed to the constituents of the diffusate to maintain life, but were in contact with some substance that was favorable to the development of hyaline necrosis. Whether this was an enzyme itself, or oxygen, or some other constituent promoting the action of an enzyme within the fibers is unknown. While the autolytic alterations in the totally ischemic center

of the muscle were slow to evolve, hyalinization of fibers near the periphery was seen after the circulation had been interrupted for only 4 to 6 hours. Since, as will be shown in a later publication, this is the same duration of ischemia that leads to irreversible changes in the muscle, hyalinization of fibers in the transitional zone between live and discoid fibers probably evolves rapidly, perhaps at the instant of death.

Fading and disappearance of red cells in the central zone of necrosis has been noted by other authors investigating infarction.[15–17] This phenomenon may be due to accumulation of acid breakdown products in the dead tissue, combined with the deficiency of oxygen.[15,16]

The emigration of leukocytes from the capillaries of the surrounding viable tissue into the ischemic muscle, and their subsequent migration into the periphery of the inner dead layer of fibers is parallel to a similar process occurring in experimental renal infarction where the infiltration reaches its height in 48 hours and does not increase thereafter. Sheehan and Davis[15,16] suggested that it was primarily a result of leukotactic irritants diffusing into the neighboring live tissue from the dead tissue, and presented evidence that the cessation of the process after 48 hours might be due to the development of a refractory state in the capillaries of the viable tissue.

The method utilized for demonstrating glycogen in the present experiment (10 per cent formalin fixation, PAS staining with diastase control) does not have quantitative validity, inasmuch as a proportion of the glycogen is undoubtedly metabolized if the muscle contracts during processing, and an additional amount is lost during formalin fixation.[18] Nevertheless, it is possible to state that the loss of glycogen from totally ischemic muscle is gradual, with stainable substance persisting 6 or more hours after the onset of ischemia. This observation is in striking contrast to that in ischemic canine cardiac muscle; here the fibers are more or less totally depleted of glycogen within 30 minutes.[19,20] Numerous authors[17,18,21,22] studying ischemia of the heart in various species have stressed the role of sustained activity of the anoxic cardiac muscle in promoting the rapid loss of glycogen. When, on the other hand, autolysis of canine cardiac muscle proceeds *in vitro* at body temperature, the depletion of glycogen approaches that of ischemic skeletal muscle *in vivo*.[20] It is assumed then that an absence of or at least greatly reduced contractile activity is responsible for the much longer persistence of glycogen in ischemic skeletal muscle.

The much greater tolerance of skeletal muscle for ischemia (4 to 6 hours before irreversible changes occur) than cardiac muscle (20 to 30 minutes before irreversible changes occur) may be related to the lessened utilization of glycogen for contraction, and its consequent greater avail-

ability as a source of energy for maintaining intracellular integrity. Although glycogen depletion is not synchronous with cell death, preceding it in both cardiac and skeletal muscle, nevertheless, *in vitro* studies have shown an inverse relationship between cellular autolysis and glycogen content in both contractile (cardiac muscle)[23] and noncontractile (hepatic) cells.[24]

The formalin fixation and Sudan IV reaction employed for demonstrating lipids does not afford an accurate quantitation of total lipid content of cells. Deane[25] has shown that this type of fixation allows progressive destruction and removal of certain lipids, especially phospholipids. Moreover, Sudan IV fails to stain phospholipids, which are known to be present in the I bands of skeletal muscle. Despite its shortcomings, the technique utilized yielded results of some interest. A great variation in the amount of lipid, ranging from none to considerable amounts, was observed not only in individual control muscles but from fiber to fiber, and from one segment of a fiber to another. Similar variations were described in feline skeletal muscle by Denny-Brown[26] who related the amount of lipid to the nutrition of the animal. Our results further showed no consistent increase in sudanophilic droplets with progressive anoxia. Published reports are rather confusing regarding the *in vitro* alterations of stainable fat in autolysis and in ischemia in the living animal. According to several observers, small granules that reduce osmic acid appear in cells undergoing autolysis,[12] but Cruickshank[12] was unable to confirm this, using both osmic acid and Sudan III. Wartman, Jennings, Yokoyama and Clabaugh[27] have demonstrated fat accumulation in myocardial ischemia in the dog as early as 4 hours after coronary ligation; observations have been variable in smaller animals.[17,18]

Results using stains for succinic dehydrogenase have confirmed those of most other investigators working with both cardiac and skeletal muscle in regard to the remarkable stability of this enzyme during ischemia.[28,29] Indeed, even after 48 hours of ischemia, considerable quantities remained.

Summary

An experimental method for producing reversible total ischemia of skeletal muscle in dogs has been described.

The histologic alterations in the muscles maintained at rest length during the onset of ischemia were those of discoid necrosis. The striation pattern of this form of necrosis was that of normal rest-length muscle and depended upon the loss of contractility of the fibers that occurred prior to death. Slowly evolving autolytic changes accentuated the discoid appearance as a result of degeneration and paling of the I band in association with retention of eosinophilia of the A band. Muscles allowed to

shorten at the onset of ischemia did not exhibit discoid necrosis, but rather a cross striation pattern of exaggerated contraction. Glycogen disappeared from ischemic skeletal muscle much more slowly than from ischemic cardiac muscle, probably because of sustained contractile activity on the part of the latter.

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LEGENDS FOR FIGURES

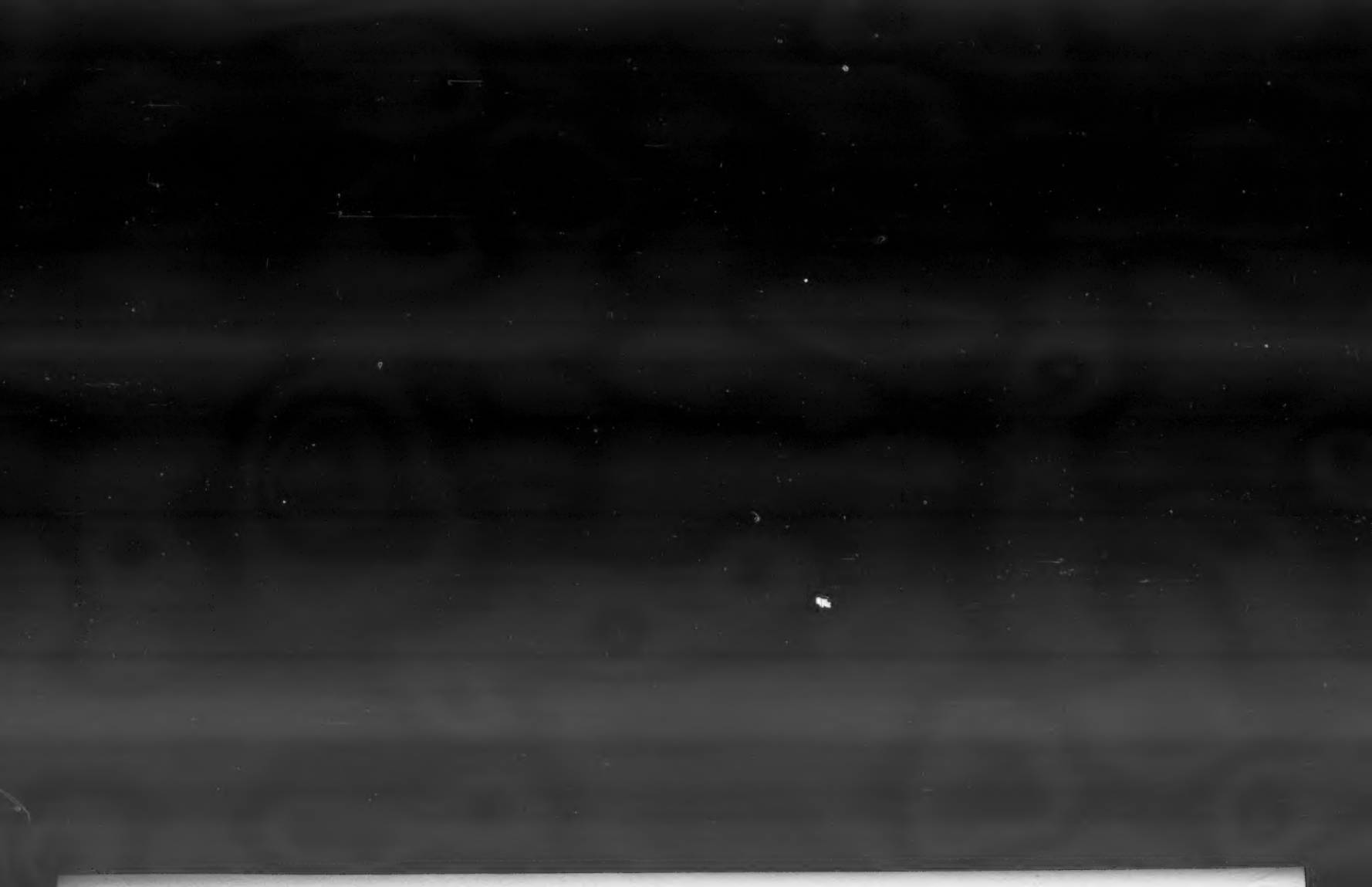
Photomicrographs were prepared from sections stained with hematoxylin and eosin.

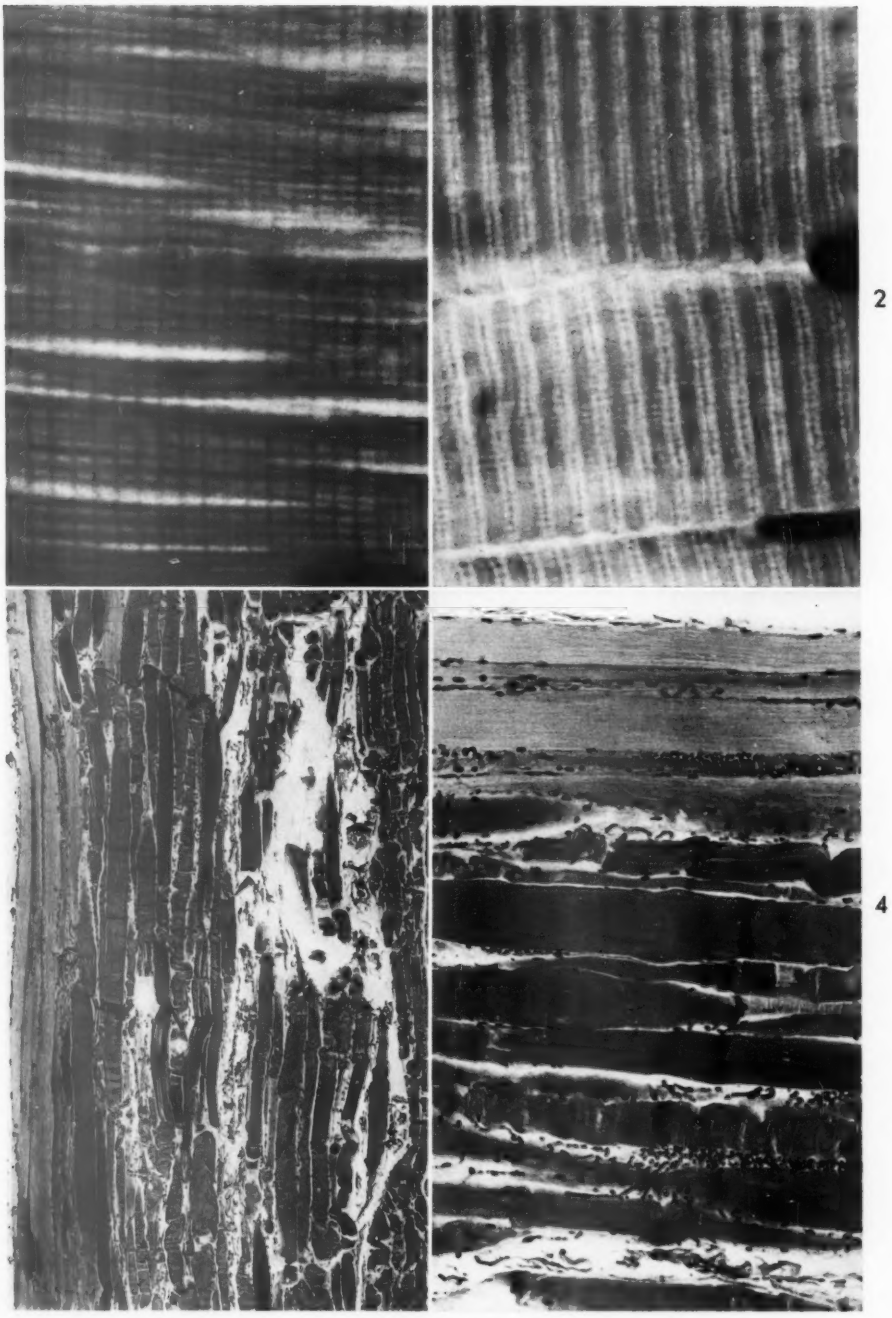
FIG. 1. Normal muscle in shortened state; note the dark contraction bands. The pale A bands are bisected by faint lines believed to represent the M lines. × 1,700.

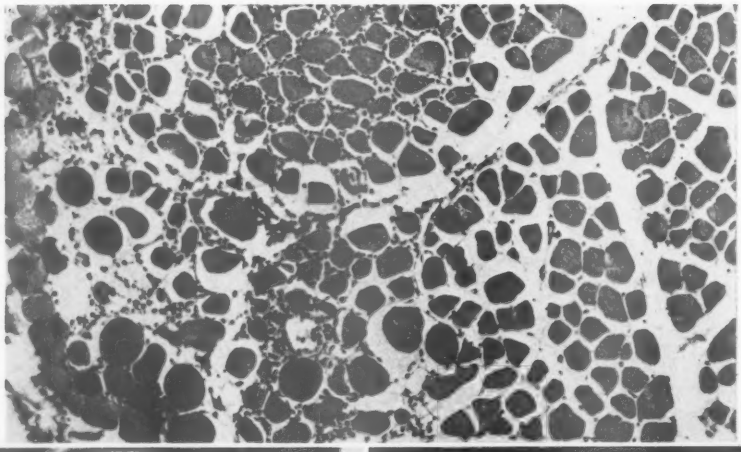
FIG. 2. Normal muscle in a rest-length state. The broad dark band is the A band; the broad pale band is the I band bisected by the delicate Z line. × 1,700.

FIG. 3. Muscle totally ischemic for 6 hours. A pale layer of normal muscle 3 to 4 fibers in thickness lies along the left margin. To the right of this layer is a layer of dark swollen hyaline fibers, 3 to 5 fibers in thickness. The bulk of the muscle is composed of dead fibers which are separated from one another. × 50.

FIG. 4. Muscle totally ischemic for 6 hours. Note the layer of normal muscle 3 to 4 fibers thick (above) and the adjacent layer of dark swollen hyaline fibers showing patchy hemorrhage. × 150.







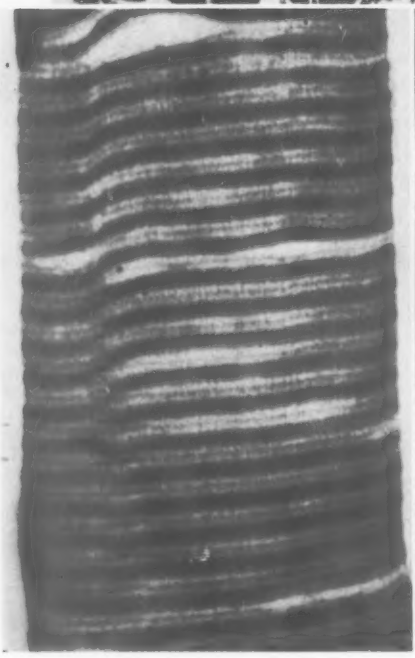


FIG. 5. Muscle totally ischemic for 10½ hours. There is a thin outer layer of normal muscle 1 to 2 fibers thick (extreme left) and a middle layer of dark swollen hyaline fibers, separated by leukocytic infiltrate. A deeper layer contains necrotic fibers that are not hyalinized, but are separated by leukocytes. The innermost mass is composed of necrotic fibers that have separated from the endomysium (extreme right). × 150.

FIG. 6. Muscle fiber totally ischemic for 24 hours. The A band stains more deeply than in normal rest-length muscle (Fig. 2), while the I band is paler. The Z line is still visible. There are areas of cracking in the I band. × 1,700.

FIG. 7. Muscle fibers totally ischemic for 48 hours. There is more widespread cracking along the I bands, and the A bands have a curved shape. × 750.

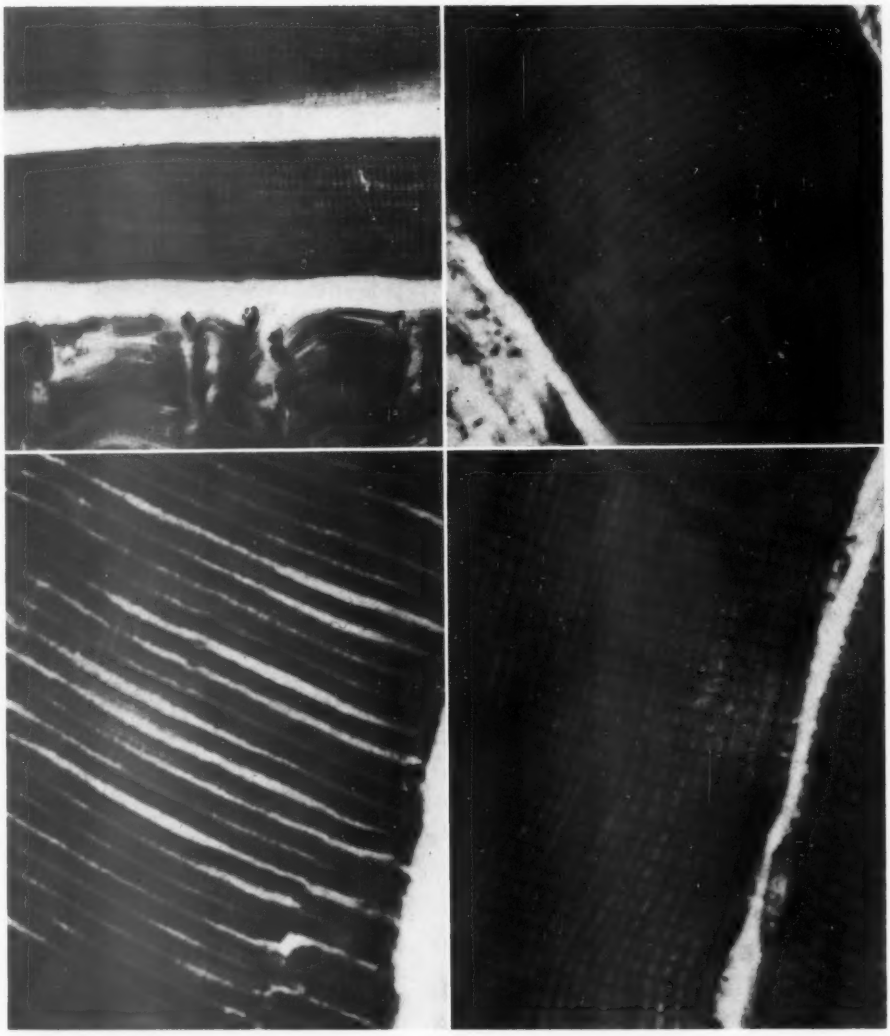


FIG. 8. Muscle fibers totally ischemic for 24 hours (tendinous ends not resutured). The lowermost fiber has a wavy character. In the remaining 2 fibers, the striation pattern of shortened muscle is apparent with prominent delicate contraction bands. × 750.

FIG. 9. Muscle fiber totally ischemic for 48 hours (tendinous ends not resutured). Delicate contraction bands are prominent, and longitudinal fibrils retain their identity. × 1,700.

FIG. 10. Muscle fiber from a case of Volkmann's contracture of 11 months' duratioñ. Dark A bands are prominent and there are empty spaces in the region of the I bands (discoid) necrosis). × 1,700.

FIG. 11. Muscle fibers from a case of Volkmann's contracture of 11 months' duration. Contraction bands are prominent. × 1,700.

THE RARITY OF INTRAHEPATIC METASTASIS IN CIRRHOSIS OF THE LIVER

A Statistical Explanation with Some Comments on the Interpretation of Necropsy Data

B. H. Ruebner M.D.; R. Green, M.D.; K. Miyai, M.D.*;
G. Caranasos, B.S.,† and Helen Abbey, Sc.D.

From the Department of Pathology, The Johns Hopkins Hospital and University, and the Department of Biostatistics, School of Hygiene, The Johns Hopkins University, Baltimore, Md.

Metastatic carcinoma is rarely seen in the liver of a patient with portal cirrhosis.[1–6] Chomet, Valaitis and Pearah reviewed the literature and found this association in only 23 cases, to which they added 3 of their own.[4] Since then, 18 further cases have been reported,[5,6] bringing the total to 44. Various explanations for the rarity of this coincidence have been put forward, but, so far, none have been generally accepted. Lisa, Solomon and Gordon[1] suggested that the cirrhotic liver may represent an unsuitable "soil" for metastatic cancer. The altered vasculature of the cirrhotic liver has also been incriminated.[3] Another possible explanation is that portal cirrhosis may be fatal before patients have a chance to develop cancer, or, should cancer supervene, patients with cirrhosis may not live long enough to develop metastasis.[3] In none of the previous studies were patients with cirrhosis compared with a control group of necropsy subjects matched for age, sex and color. Using such a control group, we have confirmed the rarity of metastatic cancer in patients with hepatic cirrhosis. By analyzing our results we have also attempted to identify the factor or factors responsible.

Material and Methods

Patients with cirrhosis of the liver were selected from 23,000 necropsies on the basis of a recorded anatomic diagnosis of portal cirrhosis, Laennec's cirrhosis, nodular cirrhosis or postnecrotic cirrhosis. Microscopic material from all these cases was rescrutinized, and those not showing definite nodular regeneration and fibrosis were excluded from this investigation. Those patients listed in the necropsy records as having cardiac or biliary cirrhosis were also excluded. In this way a group of 399 patients with hepatic cirrhosis was obtained.

A matched control group was obtained in the following way: the 23,000 necropsies were divided into 5 groups (J.H.H. necropsy No. 5,000 to 10,000; 10,000 to

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* Postdoctoral Fellow.

† Predoctoral Fellow.

15,000; 15,000 to 20,000; 20,000 to 25,000 and 25,000 to 28,000). For each patient in the cirrhosis group a control subject of the same age, sex and color was selected at random from the same group of necropsies.

All malignant tumors encountered in the cirrhosis and the control groups were noted and classified as to primary site and organs involved by metastasis. Cases of lymphoma were excluded. There were two of these among the patients with cirrhosis and 9 among the control group. Malignant tumors arising in the liver itself (hepatoma) were also excluded. Twenty-eight of these were associated with cirrhosis compared with only one which was not.

Results

The greatest majority of both groups of 399 patients, those with and those without cirrhosis, were white (73 per cent) and males predominated (68.7 per cent).

Extrahepatic Cancer in Cirrhosis and Controls

Extrahepatic cancer was present in 54 (13.5 per cent) of 399 patients with portal cirrhosis (Table I). Among the control patients there were

Table I

FREQUENCY OF EXTRAHEPATIC CANCER AND HEPATIC METASTASIS IN PATIENTS WITH AND WITHOUT CIRRHOSIS OF THE LIVER

	Cirrhosis	Controls
Number of patients	399	399
Extrahepatic cancer	54 (13.5%)	108 (27%)
Hepatic metastasis	11	39

108 cancers (27 per cent). The frequency of malignant tumors among cirrhosis patients was, therefore, exactly half of that in a matched control group. This difference was statistically significant ($X^2 = 63.5$; $p < 0.001$).

Hepatic Metastasis in Cirrhosis and Controls

Among cirrhosis patients only 11 of the 54 malignant tumors (20 per cent) had metastasized to the liver (Table I) as compared with 39 out of 108 (36 per cent) in the control group. The frequency of hepatic metastasis was thus approximately half that in the control group. This difference did not reach conventional levels of statistical significance ($X^2 = 2.1$; $0.2 > p > 0.1$). A different method of approach was, therefore, used. The total number of primary cancers at various extrahepatic sites in the entire necropsy population of The Johns Hopkins Hospital was tabulated. For each site the number of hepatic metastases was divided by the total number of cancers, and in this way a factor measuring the frequency of hepatic metastasis for each organ was obtained. The

number of cancers at each site in cirrhosis and control patients was multiplied by the appropriate factor in order to obtain the expected number of hepatic metastases. A few patients with unusual tumors such as mesothelioma and tumors of very variable malignant potentiality such as testicular cancers were excluded from this calculation. For this reason only 51 cirrhosis cases and 97 controls were included in this comparison (Table II). The actual frequency of hepatic metastasis was less than

TABLE II

EXPECTED AND ACTUAL NUMBER OF HEPATIC METASTASES IN CIRRHOSIS AND CONTROLS WITH CANCER

	51 patients with cirrhosis	97 controls
Expected number of hepatic metastases	13.7	28.8
Actual number of hepatic metastases	10	35

TABLE III

TUMOR DISSEMINATION BEYOND REGIONAL LYMPH NODES IN PATIENTS WITH AND WITHOUT HEPATIC CIRRHOSIS

	Cirrhosis	Controls
Number with extrahepatic cancer	54	108
Dissemination beyond regional nodes	18 (33.3%)	68 (63%)
Hepatic metastasis	11	39

expected among patients with cirrhosis. Again this difference did not reach statistical significance ($X^2 = 1.8$; $p > 0.5$). It seemed possible, however, that there was a true reduction in the frequency of hepatic metastasis among patients with cirrhosis and that the lack of a statistically significant difference might be due to the relatively small numbers involved. In addition, the increased frequency of hepatic metastasis in our matched control group, compared with the expected incidence, suggested that this group (and, therefore, also that with cirrhosis) was not a representative sample of the total necropsy population but differed from it in some important respect (e.g., sex, age, or date of necropsy).

Tumor Spread Beyond Regional Lymph Nodes in Cirrhosis and Controls

The possibility that cancer metastasized less widely in patients with cirrhosis than in subjects without cirrhosis was investigated next. Metastatic spread beyond the regional lymph nodes occurred in 18 of 54 (33.3 per cent) cirrhosis patients with cancer as compared to 68 of 108 (63 per cent) of the control group (Table III). This difference was statistically significant ($X^2 = 13.2$; $p < 0.001$), showing that tumor dissemina-

tion as judged by this criterion was half as frequent in cancer patients with cirrhosis as in cancer patients without cirrhosis.

Hepatic metastasis was seen in 11 of the 18 (61 per cent) cirrhosis patients with disseminated cancer (Table III). Among the control group, 39 of the 68 (57.4 per cent) metastasizing cancers involved the liver. Once a tumor had metastasized, the likelihood of liver involvement was, therefore, equal in patients with cirrhosis and in control subjects.

Frequency of Various Tumors in Cirrhosis and Controls

The distribution of malignant tumors in the group of patients with cirrhosis was very similar to that in the control group (Table IV). The

TABLE IV

DISTRIBUTION OF MALIGNANT TUMORS IN PATIENTS WITH AND WITHOUT CIRRHOSIS

	Cirrhosis	Controls
Head and neck	7	11
Lung	4	11
Breast	2	6
Other thoracic tumors	2	3
Gastrointestinal	14	30
Genito-urinary	24	45
Other abdominal tumors	0	1
Extremities	1	1
Total	54	108

relative rarity of hepatic metastasis could therefore not be attributed to a greater proportion of tumors with small propensity for metastatic spread to the liver.

Post-operative Survival in Cirrhosis and Controls

Post-operative survival after surgery for cancer was considerably shorter among cirrhosis patients than among those without cirrhosis. Only 50 per cent of the former lived more than 3 weeks post-operatively compared with a median of 3 months among the controls (Table V).

Mean Age of Patients With and Without Cancer

The mean age of patients with and without cirrhosis was 52.3 years (Table VI). In each group the age of patients with cancer was several years higher than that of patients without cancer.

DISCUSSION

The rarity of hepatic metastasis in patients with cirrhosis of the liver was undoubtedly, at least in part, due to a significant decrease in extra-

hepatic cancer in this group of patients compared with matched controls (Table I). Extrahepatic malignant tumors in cirrhosis patients were found to be rare by all previous authors who had studied this problem.[2,5–7] Fisher, Hellstrom and Fisher [6] noted that among cirrhosis patients, those without cancer were significantly younger than those with cancer, and our results corroborate this (Table VI). Unlike these authors, however, we found a similar, though somewhat smaller, age difference between those with and those without cancer in our controls also, a group which had been selected to have the same average age as the cirrhosis patients. Since there were twice as many patients with cancer among the controls, it seems likely that our matching method favored the selection of comparatively young control patients with cancer. This may account for the finding of a smaller age difference between those with and without cancer in the control group. The relatively low average age in cirrhosis without cancer, therefore, can hardly explain the diminished incidence of malignant tumors in cirrhosis of the liver.

Mainland [8] proved that the use of a control necropsy group matched for age, sex, etc., did not eliminate all statistical bias and pointed out some of the risks of drawing fallacious conclusions from such data. Cornfield [9] pointed out that even if two fatal diseases had no relationship in a living population, there would, nevertheless, be a negative association between them in a necropsy population. The necropsy incidence of one

TABLE V

DURATION OF LIFE AFTER OPERATION FOR CANCER IN PATIENTS WITH AND WITHOUT CIRRHOSIS

	No.	Median survival	Mean survival
With cirrhosis	18	3 weeks	11 months
Without cirrhosis	49	3 months	16 months

TABLE VI

MEAN AGE OF PATIENTS WITH AND WITHOUT CIRRHOSIS

	Cirrhosis	Controls
Entire groups	52.3 years	52.3 years
With cancer	61.9 years	55.7 years
Without cancer	50.4 years	50.9 years

lethal disease (e.g., cancer) among patients affected by a second fatal disease (e.g., cirrhosis) would, therefore, always be reduced in comparison with the rest of the necropsy material. The higher the mortality of the second disease, the smaller would be the proportion of patients

who, at necropsy, were affected by both diseases. A measurement of the mortality of hepatic cirrhosis is the ratio of the number of necropsied cases with cirrhosis as the only cause of death to the total number of necropsies with a diagnosis of cirrhosis. Since the definition of a cause of death is arbitrary, we reviewed only a small sample of the necropsy protocols. This suggested that more than half of the patients with cirrhosis at necropsy had no other disease which might have caused death. The mortality of hepatic cirrhosis thus seemed sufficiently high to explain the different incidence of cancer in cirrhosis and controls. Necropsy selection alone, therefore, could explain the infrequency of cancer in our cirrhosis group.

A negative relationship between cancer and other diseases has frequently been observed in the past. Pearl,[10] for instance, observed a diminished incidence of cancer in necropsy subjects with tuberculosis. It is now generally recognized[9] that this result was due to the high mortality of tuberculosis rather than to a reduced likelihood of the development of cancer in these patients. In a recent necropsy study, it was suggested that patients with diabetes had a reduced cancer incidence.[11] Another investigator,[12] however, stated that the increased mortality in diabetes was probably sufficient to account for such a result. A positive association at necropsy, such as we found in this study, between cirrhosis and hepatoma is, on the other hand, much more likely to represent a true clinical correlation. The relationship of hepatoma to cirrhosis is, of course, well recognized.

The low incidence of malignant tumors in portal cirrhosis could not alone account for the rarity of metastasis in our patients. Fisher, Hellstrom and Fisher[6] found that patients with hepatic cirrhosis were unusually prone to develop tumors of the head and neck, which have little propensity for metastasis. This factor did not play a part in the infrequency of hepatic metastasis in our cirrhosis group since the distribution of the various types of cancer in these patients closely resembled that among the control subjects (Table IV). The proportion of malignant tumors metastasizing to the liver in our cirrhosis patients was approximately half that among the control group (Table I). Calculation of the expected number of hepatic metastases led to a similar conclusion: namely, that in cirrhosis hepatic metastases were less frequent than in the general necropsy population (Table II). This method of calculation did not permit the use of controls matched for age, sex, color and date of necropsy. Although these results were suggestive, neither method yielded a statistically significant difference, probably because of the relatively small numbers involved. The investigation, therefore, was extended to include not only hepatic metastases but also tumor dissemination to other

parts of the body (Table III). Tumor spread beyond the regional lymph nodes in the cirrhosis patients with cancer was found to be significantly less frequent than in the cancer patients of the control group. Once the tumor had spread beyond the regional lymph nodes, however, hepatic metastasis was as frequent in those with as in those without cirrhosis. A study of patients who were operated upon for cancer (Table V) confirmed a general clinical impression that patients with hepatic cirrhosis had a higher post-operative mortality than those without cirrhosis. No doubt this is one of the factors preventing cirrhosis patients with cancer from living long enough with their tumors to develop metastasis. Thus the high mortality of cirrhosis appears to be responsible for the reduced tendency to metastasis of malignant tumors as well as for the rarity of cancer in this group.

Patients with myocardial infarction resemble those with disseminated cancer in having a high mortality. We have shown [13] that the incidence of myocardial infarction in patients with hepatic cirrhosis is decreased.[14,15] The frequency of both disseminated cancer and of myocardial infarction among individuals with cirrhosis was approximately a quarter of that among matched controls. This similarity is to be expected if necropsy subjects affected by one fatal disease (e.g., cirrhosis) tend to have a reduced incidence of any second fatal disease (cancer or myocardial infarction) when compared with the general necropsy population.

Our investigation has provided no evidence that the cirrhotic liver represents an infertile "soil" for metastatic cancer or that the altered vasculature of the cirrhotic liver is responsible for the rarity of hepatic metastasis.[3] There is no doubt, however, that experimentally the susceptibility of the liver to metastasis can be influenced,[16] and further study of this problem by appropriate methods is indicated.

Summary

The rarity of hepatic metastasis in patients with cirrhosis of the liver could be accounted for by two factors: firstly, a significant reduction in the frequency, at necropsy, of extrahepatic cancer in individuals with cirrhosis; secondly, a decreased tendency to metastasis in cirrhosis patients who developed malignant neoplastic disease. Tumor dissemination to the liver in patients with cirrhosis was diminished to the same extent as spread to other organs.

It is pointed out that the necropsy incidence of one lethal disease (e.g., cancer) will generally be diminished among patients with a second fatal disease (e.g., cirrhosis). We consider that the high mortality of cirrhosis is probably responsible for the diminished tendency of malignant tumors to metastasize as well as for the infrequency of cancer in this group.

Our investigation has produced no evidence that the cirrhotic liver represents an unfertile "soil" for metastatic cancer or that the altered vasculature of the liver is responsible for the rarity of hepatic metastasis.

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